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STUDIES ON THE CUTICLE OF THE  
HUMAN BODY LOUSE, PEDICULUS HUMANUS HUMANUS.

BY

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JANUARY 1982.



NUMEROUS ORIGINALS  
IN COLOUR



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## Abstract.

The cuticular ultrastructure and surface topography of the human body louse, Pediculus humanus humanus, were examined. Under light microscopy, pore canals were readily observed in unstained, cryostat sections of cuticle from various locations on the insect, particularly those areas composed of smooth, sclerotised cuticle. Transmission electron microscopy (TEM) studies revealed that, after attachment at the epidermis, the canals appeared to pass unbranched through the endocuticle (average thickness 4.5 $\mu$ ) with diameters around 1000 $\text{\AA}$ . Branching occurred in the exocuticle (thickness 1.0 $\mu$ ) and finally in the epicuticle (thickness 2000 $\text{\AA}$ ) to form wax canals with diameters around 100 $\text{\AA}$ . Pores or openings with diameters around 100 $\text{\AA}$  were found during scanning electron microscopic (SEM) studies of the epicuticular surface and corresponded with the wax canals noted in the TEM examinations.

A chemical analysis of the cuticular lipids of the louse was conducted using thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and gaschromatography/mass spectroscopy (GC/MS) and the hydrocarbons were found to be the most abundant class (almost 75 per cent). Cholesteryl esters, triglycerides, 1,3-diglycerides, cholesterol and fatty acids were also present in that order of abundance. Cuticular hydrocarbons were comprised of over 43 components which contained from 21 to 35 total carbons per molecule. Four homologous series were present and consisted of n-alkanes (51.8 per cent of total cuticular hydrocarbons), n-alkenes (26.4 per cent), internally branched monomethylalkanes (12.1 per cent) and a tentatively identified series comprising dimethylalkanes (4.7 per cent).

The effect of insecticide solvents upon the cuticular wax layer was investigated in vitro by comparing the solubility of a simulated cuticular wax mixture in a series of ten potential solvents. Results are discussed along with those from toxicity tests performed with the solvents on adult female lice and indicate a relationship between cuticular wax solubility and louse mortality. Studies with

a fluorescent dye, demonstrated that wax-dissolving solvents entered the insect via at least two routes, i.e. cuticle and spiracles.

The results are compared with the published work on other insect cuticular systems and the implications involving potential insecticide carriers discussed.

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## 1. Introduction and Literature Review.

### 1.1. Structure of arthropod cuticle.

Arthropod cuticle is a remarkably complex, composite material which acts not only as the interface or barrier between living animal and environment but also as an exoskeleton. Early investigators could not have envisaged the true extent of their task in attempting to define the nature of arthropod cuticle and its intrinsic functions. Although it is generally recognised that modern studies on cuticle began with K hnelt (1928 a, b, c) and Wigglesworth (1933), significant advances were made throughout the nineteenth century in the recognition and recording of basic aspects of cuticle architecture. With the continuous refinement of techniques since this period (and becoming highly intensified over the past three decades) the study of arthropod cuticle has been, and remains, a rich field for scientific research, involving many disciplines. Numerous publications have been presented through the years, either as papers on specific topics related to arthropod cuticle, or as short general reviews (Wigglesworth, 1948, 1957, 1965; Hackman, 1971; Looke, 1974); but, remarkably, only two books devoted entirely to the comprehensive review of cuticle biology (Richards, 1951; Neville, 1975) have been produced. In recent years the widespread interest in the field has been reflected in the appearance of collections of monographs in book form on various aspects of arthropod cuticle (Hepburn, 1976; Gilby, 1980; Locke and Smith, 1980).

Perhaps the earliest evidence of the composite nature of arthropod cuticle was provided by Odier (1823) who discovered and named chitin in the elytra of the May beetle and also recognised the presence of protein. In the same publication, he noted that the outermost layer of the cuticle was composed of a thin sheet of highly refractile material and this corresponds to the structure now known as the epicuticle. Thick cuticle from arthropods such as beetles was particularly useful for examination with the microscopes of the period. Observations by Meyer (1842) on the elytra of the stag beetle Lucanus revealed the lamellate architecture of the endocuticle and the existence of lateral strands (balken) embedded within the homogeneous matrix of the layer. Although this fibrous cross-ply structure is restricted to arthropods such as beetles and crustaceans, Meyer's discovery helped to establish the concept of fibres as being the basic constructional units of cuticle in general. As microscopy improved and with the development of techniques involving fluorescence and polarised light, this concept was consolidated. The phenomenon of form birefringence observed in purified chitin (Clark and Smith, 1936) was believed to demonstrate the presence of parallel microfibrils or micelles which formed the larger fibres. Much higher resolution was achieved with the development of the electron microscope and Richards and Korda (1948, 1950) were able to observe chitin microfibrils which they presumed to be the basic structural units (chitin micelles). X-ray diffraction studies to determine the molecular conformation and structure of chitin (Fraenkel and

Rudall, 1940, 1947; Lotmar and Picken, 1950) were also undertaken during this period, but in many cases samples of isolated chitin were contaminated by residual protein which caused results to be misinterpreted.

Using Crustacean cuticle, Bouligand (1965) demonstrated 250 Å diameter fibrous components which he regarded as the basic structural units of chitin. However, Rudall (1965, 1967) observed even smaller components (now termed microfibrils or crystallites) with diameters from 25-60 Å in the ovipositors of adult Sirex and larval and puparial cuticle of Sarcophaga. An early model (Fraenkel and Rudall, 1947) which proposed that cuticle is constructed of alternating monomolecular layers of chitin and  $\beta$ -extended protein was later challenged by Jensen and Weis-Fogh (1962) who concluded that such a system would be co-crystalline in structure and extremely brittle whilst the chitin concentration in the model would be uncommonly high. Instead, they regarded cuticle as a composite in view of their results from mechanical experiments with locust cuticle. This interpretation was supported by Rudall (1967) who suggested that the basic cuticle system consisted of chitin microfibrils embedded in a protein matrix. Using an improved staining technique which took advantage of the different reactions of chitin and protein with potassium permanganate and lead citrate, Neville and Luke (1969b) were able to achieve, using the electron microscope, a very high contrast between the two cuticle components. Neville (1970) and Weis-Fogh (1970) subsequently demonstrated that microfibrils in



the locust prealar arm ligament are composed of chitin and lie in a protein matrix. On the basis of X-ray diffraction studies, Rudall and Kenchington (1973) estimated that the chitin molecule chains in each microfibril are arranged in 3 layers with 7 chains per layer. This was later revised to 3 layers of 6 chains by Neville (1975) who used an average microfibril diameter of  $28 \text{ \AA}$  (Neville, Parry, and Woodhead-Galloway, 1976) in calculating numbers of molecule chains per chitin microfibril. In several instances, optical diffraction patterns from electron micrographs of microfibril/matrix systems have indicated that the microfibrils are themselves arranged hexagonally in the protein matrix (Neville *et al.*, 1976).

The lamellate appearance of arthropod cuticle has been the subject of considerable discussion over the years and, whilst the suggestion that lamellae allow for flexibility and stretching of the cuticle by sliding over one another (Ahrens, 1930) found wide acceptance (Wigglesworth, 1965), it was strongly rejected elsewhere (Richards, 1951). Richards (1951) interpreted the lamellae of insect procuticle as representing alternating layers of dense and less dense material. Examination of oblique sections of insect cuticle in the electron microscope revealed, however, that parabolic patterning exists between the lamellae (Locke, 1960) similar to that seen in light microscope observations on Crustacean cuticle (Drach, 1939, 1953; Dennell, 1960) which generally possesses thicker fibrous components. The theory that such patterning results from continuous arcs between the lamellae

(Drach, 1953; Dennell, 1960, Locke, 1974) was disproved by Bouligand (1965, 1972) who proposed instead, that helicoidal architecture of the cuticle is responsible. Bouligand (1965, 1972) concluded that lamellate cuticle is formed by successive microfibril planes which are parallel to one another and to the cuticle surface. The direction of microfibril orientation in each plane differs from the preceding one by a small angle and always in the same direction. Such a formation had been suggested much earlier by Michelson (1911) whose observation that the cuticles of some arthropods reflect circularly polarised light prompted him to speculate that a screw-type of structure might be involved.

As pore canals traverse the chitin/protein complex of the cuticle, their shapes (as seen from the surface) reflect the orientation of the successive microfibril layers. Pore canals were widely believed to follow a helical course (Richards and Andersen, 1942; Wigglesworth, 1933, 1948; Locke, 1957) but later observations with both the light (Neville *et al.*, 1969) and electron microscope (Neville and Luke, 1969a) revealed that they behave in a ribbon-like manner by rotating in helicoidal layers and remaining untwisted in layers of unidirectional microfibrils. On the basis of pore canal shapes and orientation in locust cuticle sections, Neville and Luke (1969a, b) formulated a two-system model for cuticle architecture in arthropods whereby layers consist of chitin microfibrils arranged either helicoidally in the protein matrix or unidirectionally to form a preferred layer.

## 1.2. Cuticular lipids of arthropods.

Most terrestrial arthropods examined to date possess a wax layer on the epicuticle which is considered to be primarily responsible for minimising the transpirational water loss through the integument (Etheling, 1974; Jackson and Baker, 1970; Edney, 1974). The role of cuticular waxes in the waterproofing of arthropods was first reported by Ramsay (1935) when he observed that a rapid increase in water loss from the cockroach occurred above a critical temperature (about 35°C for the cockroach). Later, Wigglesworth (1945) obtained similar results using a wider range of insects; whilst Beament (1945) showed that there was general agreement between the critical temperatures recorded by Wigglesworth from whole insects and those produced by waxes extracted from the same insects and deposited on artificial membranes. Chemical analyses of cuticular waxes during this period were, at best, rudimentary; however, the advent of gas-liquid chromatography (GLC) in the early 1950s followed by thin-layer chromatography (TLC) at a later stage revolutionised organic analytical chemistry in that it became possible to resolve lipid mixtures into their individual components using small (submilligram) amounts of sample. Currently, the highest levels of performance in terms of resolution and identification of lipids is achieved by using a capillary column GLC and a mass spectrometer (MS) in an integrated system (GC-MS) linked to a data processing unit.

The earliest reports on arthropod cuticular lipids, which involved the use of chromatographic techniques, were published in the 1960s and include descriptions of lipids from the Mormon cricket Anabrus simplex (Baker *et al.*, 1960), the cockroach Periplaneta americana (Baker *et al.*, 1963; Gilby and Cox, 1963) and larvae of the silkworm Samia cynthia ricini (Bowers and Thomson, 1965). Since then the cuticular lipid compositions of many arthropod species have been examined and the results comprehensively reviewed by various authors (Jackson and Baker, 1970; Hackman, 1974; Neville, 1975; Jackson and Blomquist, 1976; Blomquist and Jackson, 1979; Hadley, 1981). In addition, specific groups of lipids such as the cuticular hydrocarbons (Lockey, 1980a) and long-chain methyl-branched hydrocarbons (Helson, 1978) have also formed the basis of detailed reviews. Extensive accounts of recent techniques in insect lipid analysis have been provided by Jackson and Arnold (1977) and Gilby (1980).

Among the most common components of arthropod cuticular lipids are hydrocarbons, free fatty acids, triglycerides, and sterols whilst wax esters, alcohols and aldehydes are also occasionally present. Because hydrocarbons have been identified in all cuticular extracts investigated to date and often comprise the most abundant lipid class (Jackson and Blomquist, 1976; Lockey, 1976), they are assumed to play an essential role in the water-proofing of arthropods (Hadley, 1978). Cuticular hydrocarbon

content in each of five species of desert tenebrionid beetles exceeds 90% of total lipids (Hadley, 1978) and similar levels have been reported in seven cockroach species (Jackson, 1970, 1972; Tartivita and Jackson, 1970). Consequently, many of the investigations carried out on cuticular lipids have been concerned only with the composition of the hydrocarbon fraction. There are numerous exceptions, however, where the hydrocarbon content of cuticular lipids is substantially lower than the levels (50 - 75%) suggested by Nelson et al., (1971) and include six species of tenebrionid beetles (< 10%) which infest stored products (Lockey, 1978a, b, 1979).

Although arthropod cuticular hydrocarbons represent a vast and complex series of compounds, they may be classified according to the following categories: n - alkanes, alkenes, monomethylalkanes with terminal branching, monomethylalkanes with internal branching, dimethylalkanes and trimethylalkanes. n - Alkanes, particularly those with an odd number of carbon atoms, are always present in arthropod cuticular hydrocarbons and n - tricosane, n - pentacosane, n - heptacosane and n - nonacosane are often the most abundant (Lockey, 1980a). Exceptions include the termite Zootermopsis angusticollis and the larval cigarette beetle Lasioderma serricorne in which the major n - alkanes are n - heneicosane (Blomquist et al., 1979a) and n - tritriacontane (Baker et al., 1979b) respectively. Hadley (1978, 1981) has proposed that maximum cuticular impermeability is most likely to be achieved with a wax containing

a high proportion of saturated, long-chain compounds. Since it appears that arthropods in general are unable to synthesise n - alkanes greater than 33-35 carbons (Jackson and Blomquist, 1976), Toolson and Hadley (1977) have suggested th that an increase in the concentration of branched methylalkanes containing more than 35 carbons might produce an overall decrease in permeability; albeit less effectively than that expected from comparable n - alkanes.

Research into the biosynthesis of cuticular hydrocarbons has been rather limited when compared with studies on composition of cuticular hydrocarbons in arthropods. Nevertheless, Nelson et al. (1971) noted a possible correlation between dietary n - alkanes and integumentary n - alkanes of the tobacco hornworm Manduca sexta which was later supported by the findings of Blomquist and Jackson (1973) who included labelled n - alkanes in the diet of the grasshopper Melanopus sanguinipes to demonstrate that a significant proportion of cuticular n - alkanes could be derived from the diet. There is also evidence indicating that n - alkanes are products of biosynthesis. Labelled acetate was readily incorporated into all cuticular hydrocarbons of the cockroach Periplaneta fuliginosa (Blomquist and Kearney, 1976) and the termite Zootermopsis angusticollis (Blomquist et al., 1979b) whilst labelled palmitate was preferentially incorporated into the n - alkanes of Z. angusticollis (Blomquist et al., 1979b). Results of experiments with cockroaches

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(Major and Blomquist, 1978) indicate that biosynthesis of cuticular hydrocarbons occurs via an elongation - decarboxylation pathway whereby 16 and 18 carbon fatty acids are the precursors of longer chain fatty acids which are converted to alkanes by a decarboxylation step.

Although unsaturated hydrocarbons, mainly n - alkenes and n - alkadienes, occur extensively throughout arthropod cuticular lipids, they are usually only minor constituents (Jackson and Blomquist, 1976). Unsaturated cuticular hydrocarbons feature predominantly in four of the seven cockroach species investigated to date (Jackson, 1972). Cis - 9 - nonacosene and cis, cis-6, 9-nonacosadiene are the major cuticular hydrocarbons of Periplaneta japonica (Jackson, 1972) whereas cis-6, 9-heptacosadiene<sup>cis</sup> accounts for half the total cuticular lipids of P. americana (Gilby and Cox, 1963; Beatty and Gilby, 1969; Jackson, 1972). Cis - 9 - tricosene is the major cuticular hydrocarbon of P. australasiae and P. fuliginosa adult males, but is only a very minor component of adult female cuticular hydrocarbons (Jackson, 1970) and a similar disparity occurs in the case of cis-9-heptacosene for adults of P. japonica (Jackson, 1972). Few reports on biosynthesis of unsaturated cuticular hydrocarbons have been published; however, cis, cis-6, 9-heptacosadiene, the major cuticular lipid of P. americana, is thought to be produced in post-ecdyial adults via a condensation - decarboxylation pathway in which linoleate is a precursor (Jackson and Baker, 1970; Conrad and Jackson, 1971).



Long-chain methyl-branched hydrocarbons have been detected only comparatively recently in arthropod cuticular lipids since their identification usually requires the use of a GC-MS system. Monomethylalkanes may be divided into two groups; terminally branched monomethylalkanes comprising 2 - methyl, 3 - methyl and 4 - methylalkanes and internally branched monomethylalkanes in which the methyl branched is positioned centrally on the carbon chain. As a class of hydrocarbons, monomethylalkanes occur almost universally throughout the cuticular lipids of arthropods and in some instances, such as the adult cigarette beetle Lasioderma serricornis (Baker *et al.*, 1979b) and the desert tenebrionid beetle Eleodes armata (Hadley, 1977), account for as much as 8% and 92.% respectively of the total cuticular hydrocarbons. Overall, 3-methylalkanes have been reported in over twice as many cuticular lipid mixtures as 2 - methylalkanes and 4 - methylalkanes have been identified only in the adult cigarette beetle Lasioderma serricornis (Baker *et al.*, 1979b). Although 2 - methylalkanes are usually only minor hydrocarbon components when present, the crickets Acheta domesticus, Gryllus pennsylvanicus and Nembius fasciatus (Blomquist *et al.*, 1976) and the beetle Phyllobius maculicornis (Jacob, 1977) are uncommon in each having a 2 - methylalkane series which accounts for 20-30% of their respective cuticular hydrocarbons. When present in cuticular lipids, 3 - methylalkanes usually comprise a reasonable percentage of the hydrocarbons. The fire ants Solenopsis invicta

and S. richteri have levels of 3 - methylalkanes in their cuticular hydrocarbons which, at 39% and 34% respectively (Lok et al. 1975), are among the highest reported for arthropods.

Internally branched monomethylalkanes have been identified in the majority of cuticular hydrocarbon mixtures investigated and usually form an appreciable proportion of the total. The cockroaches Periplaneta brunnea, P. fuliginosa (Jackson, 1970), Leucophaea maderae and Blatta orientalis (Tartivita and Jackson, 1970) have particularly high concentrations of internally branched monomethylalkanes comprising over half of each cuticular hydrocarbon mixture whereas these monomethylalkanes are absent in P. americana (Jackson, 1972). In general, internally branched monomethylalkanes containing even numbers of carbon atoms are more prevalent than those with odd carbon numbers and the methyl branch is usually located on an odd-numbered carbon atom (Nelson, 1978). They often occur as members of isomeric mixtures in which carbon numbers are identical and the methyl branch is located on different odd-numbered carbon atoms. Adults of the cigarette beetle Lasioderma serricorne (Baker et al. 1979b) have unusual cuticular hydrocarbons which contain internally branched monomethylalkanes of odd and even carbon numbers as well as mixtures of isomers with methyl branches on odd- and even - numbered carbon atoms as in mixtures of 10-, 11-, 12- and 13-methylhexacosanes. Similar situations have been observed in surface hydrocarbons from several species of tenebrionid beetles (Lockey, 1978b, 1979, 1980b).

Although dimethylalkanes occur less frequently in cuticular lipids than monomethylalkanes, they generally have the longer chain lengths (Hadley, 1981). Isomeric mixtures of internally branched dimethylalkanes are common and are usually composed of molecules with methyl branches on odd-numbered carbon atoms separated by three carbon atoms referred to as isoprenoid spacing (Lockey, 1980a). The cricket Gryllus pennsylvanicus has a relatively high proportion of internally branched dimethylalkanes (34%) with typical structures containing odd carbon numbers from 33 to 39 inclusive (Blomquist et al. 1976). A number of unusual dimethylalkanes have been identified including a series of 11, 12- isomers from the larval cigarette beetle Lasioderma serricorne which contain 33, 34, 35 and 37 carbon atoms (Baker et al. 1979b). Blomquist et al. (1979a) detected a unique cuticular dimethylalkane, 5, 17- dimethylheneicosane, in all castes of the termites Zootermopsis angusticollis which comprised about one quarter of the hydrocarbons.

In comparison with other cuticular hydrocarbons, trimethylalkanes are extremely rare. All of those identified so far have shown isoprenoid spacing of the methyl branches such as 11, 15, 19 - and 13, 17, 21 - trimethylpentatriacontanes from the grasshopper Schistocerca gregaria (Nelson and Sukkestad, 1975). Martin and MacConnell (1970) identified two homologous series based on 3, 7, 11- and 4, 8, 12 - trimethylalkanes in the whole body hydrocarbons of the ant Atta colombica. Collectively, the trimethylalkanes formed the largest fraction within the extracted hydrocarbons which

were considered to be mostly cuticular in origin.

A number of studies on the biosynthesis of branched cuticular hydrocarbons have been conducted based on the incorporation of labelled substrates in surface hydrocarbons. Results from experiments using the cricket Nemobius fasciatus indicate that valine is incorporated into 2 - methylalkanes with odd numbers of carbon atoms after conversion to isobutyric acid (Blalock et al., 1976). Similarly, leucine is incorporated into the cuticular 2-methylalkanes with even carbon numbers of the cricket Gryllus pennsylvanicus following conversion to isovaleric acid (Blalock et al., 1976). Labelled propionate was preferentially incorporated into the 3 - methylalkanes of the cockroaches Periplaneta americana and P. fuliginosa as well as the internally branched monomethylalkanes of the latter (Blomquist et al., 1976; Blomquist and Kearney, 1976). Data from trials on the termite Zootermopsis angusticollis indicate that the methyl branches of mono - and dimethylalkanes are derived from incorporation of propionate in place of acetate during chain elongation. (Blomquist et al., 1979b).

Although triglycerides, free fatty acids, and sterols are seldom major components of, arthropod cuticular lipids, their presence in the majority of these surface lipids investigated so far suggests that they (along with hydrocarbons) are in some way involved in the waterproofing process.

The surface lipids of the naiad big stonefly Pteronarcys californica contain an exceptionally high proportion of triglycerides (78%) resulting in a lower melting mixture than that of the adult which has a greatly reduced triglyceride content (7%) (Arnold et al. 1969). Qualitative and quantitative differences in the surface lipids of the aquatic naiad and terrestrial adult forms of P. californica appear to vary with environmental need for water conservation (Arnold et al. 1969).

Palmitic acid (16:0) and oleic acid(18:1) are usually the predominant free fatty acids in arthropod cuticular lipids (Jackson and Blomquist, 1976) but changes in free fatty acid composition with stages in insect development have been reported. Whilst free fatty acid content of the cuticular lipids from the larval and adult black carpet beetle Attagenus megatoma remained fairly constant during development, a significant increase in unsaturation accompanied by a general reduction in chain length was observed in adult fatty acids (Baker et al. 1979a). In comparing cuticular permeability and cuticular lipid composition of two scorpions Hadrurus a. arizonensis and Uroctonus apacheanus. Toolson and Hadley (1977) recognised among free fatty acids inverse correlations between permeability and extent of saturation and between permeability and molecule size.

Cholesterol is usually the single most abundant component (>75%) in cuticular sterols although its concentration in total

cuticular lipids is generally low. The steroid nucleus cannot be synthesised in arthropods (Gilbert, 1967) and since cholesterol is essential for development in many insect species (Vroman et al. 1964), it (or precursors) must be acquired through the diet.

Involvement of cuticular lipids in various functions apart from maintaining the water balance in arthropods has been reported in numerous species (Neville, 1975; Jackson and Blomquist, 1976; Nelson, 1978). On the basis of their effects on cultures of Aspergillus flavus, surface lipids from larvae of the silkworm Bombyx mori have significant antifungal properties, with saturated, medium-chain free fatty acids being the most potent components (Koidsumi, 1957). Examples of surface lipid components from insects acting as kairomones and sex attractants are widespread and include many long-chain alkanes which are either unsaturated or have methyl branches (Nelson, 1978).

### 1.3. The penetration of arthropod cuticle.

Studies on the permeability of arthropod cuticle to chemicals have become increasingly significant in recent years in view of the widespread use of chemical toxicants in the control of insects which pose a threat to man. Despite intensive research during the past few decades however, the mechanics of cuticular penetration are still not clearly defined. Early researchers such as Richards (1951) recognised the complexity of the field and the need for unambiguous quantitative data in attempting to understand the phenomenon, but many investigations have been qualitative in approach and conflicting observations have been published. In addition, experimental data from studies in which the existence of a direct relationship between rates of intoxication and penetration was assumed are of limited value since various physiological interactions have been found to occur when chemical mixtures are used (Brattsten and Wilkinson, 1977). In spite of the considerable interest in the field, only two major reviews have appeared to date. Eteling (1974) published a comprehensive review of work carried out up until 1974 whilst Lewis (1980) has compiled a detailed account of the various techniques which may be used in studying cuticular penetration with an emphasis on producing quantitative data.

While the effectiveness of the epicuticular wax in minimising water losses from arthropods is well established (Edney, 1974), its hydrophobic or lipophilic character is also responsible for the resistance of the cuticular surface to wetting by external water.

In contrast, non-polar liquids, which are not usually encountered by insects in their natural environment, readily wet the cuticular surface and cause extensive disruption of the epicuticular wax layer (Ebbling, 1976). Insecticidal formulations are usually prepared with this in mind and the toxicant is presented to the insect as a solution in a non-polar solvent. Some controversy exists regarding the main route of entry for contact insecticides, but most evidence suggests that penetration occurs via the pore canals and haemolymph (Wigglesworth, 1942; Lewis, 1965, 1980; Burt and Lord, 1968) rather than the tracheae (Gerolt, 1969, 1970).

Histological studies by Wigglesworth (1942) on the fifth stage nymphs and adults of Rhodnius prolixus demonstrated that the pore canals and dermal gland ducts could facilitate the passage of oils through the cuticle and this was the earliest concrete evidence for penetration via the cuticle. More recently, Bruck and Komniok (1971) observed, using the electron microscope, penetration of the epicuticular filaments and pore canals of the cockroach Periplaneta americana by an inorganic phosphate; while Lewis (1980), using autoradiographic techniques, has demonstrated absorption of [ $^3$ H]-oleic acid through the pore canals in the antenna of the locust Schistocerca gregaria. Much of the experimental evidence in support of insecticide entry via the pore canals/haemolymph route is indirect. Using the blowfly Phormia terraenovae, Lewis (1965) compared the quantities of DDT absorbed at various parts of the body which were representative of a broad spectrum of cuticular porosity. After a period of 48 hours, the antennae, which possess the most porous cuticle, had absorbed the greatest quantity of DDT per unit area



followed by the coxae and sternal-coxal membranes and finally the lens of the compound eye which possesses no pore canals. This demonstrated that not only did penetration proceed much faster in cuticle where pore canals were present, but also that penetration could still occur in cuticle in which pore canals were absent.

In the same publication, Lewis reported that absorption of DDT from sparsers of lanoline applied to the head of the blowfly occurred initially at a rapid rate which declined steeply thereafter. Although the rate of absorption into dead flies became negligible after 30 hours, the absorption rate into living flies eventually reached a steady value. Lewis suggested that the high initial absorption rate for DDT was caused by a high concentration gradient across the cuticle and the steady decline in absorption rate resulted as the concentration gradient was diminished by the accumulation of insecticide in cells adjacent to the treated area of cuticle. In living flies, circulating haemolymph was partly responsible for the final steady rate of absorption; whereas, in dead flies, a state of equilibrium was reached as the concentration gradient of DDT across the cuticle approached zero. Bi-phasic absorption in insects has also been observed with several other insecticides including malathion (Matsumura, 1963), famphur and dimethoate (Buerger and O'Brien, 1965) and isomers of benzene hexachloride (Kurihara <sup>et al</sup>, 1970), and a number of investigators (Hoffman et al, 1952; Le Roux and Morrison, 1954; Matsumura, 1963) have also found that entry rates of insecticides for dead insects are significantly lower than those for living insects.

In studies using the radioactively labelled insecticide, [<sup>14</sup>C]-DDT on houseflies Musca domestica, Le Roux and Morrison (1954)

detected radioactivity in the haemolymph of the cervical region within 30 seconds of topical application of the labelled DDT in benzol to the tibiofemoral membrane of a metathoracic leg. Benezet and Forgasz (1972) detected radioactivity in the haemolymph of houseflies within 15 seconds of topically applying thoraces with solutions of [ $^{14}\text{C}$ ]-malathion in acetone. A further indication of the involvement of haemolymph in the transportation of toxicants was demonstrated by Burt et al. (1971) using the cockroach P. americana. Exposed abdominal nerve cords in untreated insects were irrigated with haemolymph from insects poisoned with doses ( $\text{LD}_{95}$ ) of pyrethrin I. Electrophysiological monitoring of the cords revealed that after 10 minutes, spontaneous activity in sixth abdominal ganglia increased tenfold.

On the basis of the following experimental observations, Gerolt (1969, 1970) proposed an alternative theory to the widely held view that insecticides traverse the cuticle via the pore canals and are transported to the site of action by the haemolymph. When he deposited dieldrin in beeswax at various loci on the body of the housefly M. domestica, the effect was greatest at sites near the central nervous system (CNS) and least at the more distant loci. Gerolt maintains that if the insecticide is transported to the CNS by the haemolymph, distance between CNS and site of topical application would have little effect.

Although a reduction was noted in the speed of action of various insecticides upon houseflies covered in a thin film of olive oil, no reduction was observed for flies containing injections of the oil. Insecticides used in the experiment were highly soluble

in olive oil and would be expected to be readily absorbed by the internal deposits.

Houseflies which were exposed to a surface deposit of dieldrin (0.2  $\mu\text{g}/\text{fly}$ ) suffered complete knockdown in 65 minutes whereas the same mass of crystalline dieldrin introduced into the abdominal cavity produced no obvious effects in the flies during the initial 7 hours. The organophosphate insecticides, chlorphen-vinphos, methyl parathion and dichlorvos produced similar results.

When [ $^{14}\text{C}$ ]-dieldrin in benzene was topically applied to isolated insect integument mounted in a diffusion cell, no accumulation of radioactivity was detected in the underlying saline solution over a 24 hour period. Integuments from various insects were tested in the cell.

In houseflies, a band of beeswax around the 'waist' of an insect appeared to prevent insecticide (dieldrin) applied to the abdomen from migrating to the thorax by the cuticular route. Paralysis was observed among flies without wax barriers soon after treatment with an equivalent dose. Cockroaches with sealed abdominal spiracles and wax barriers displayed no adverse effects for 3 days after application of dieldrin crystals to their abdomens; but those without any protection were paralysed within 6 hours and those with wax barriers only were affected within 17 hours.

Autoradiographic investigations in which [ $^{14}\text{C}$ ]-dieldrin was applied to the abdomens of locusts Schistocerca gregaria showed that the insecticide penetrated into the deeper layers of the integument. Gerolt suggested that the dieldrin was concentrated

in the endocuticle-hypodermis region and proposed that migration of the insecticide proceeded laterally in the integument into the tracheae.

After housefly larvae were exposed to a dry deposit of [ $^{14}\text{C}$ ]-dieldrin for various periods of time, the tracheal trunks and other organs were dissected out and subjected to autoradiography. Although the distribution was uneven, some labelled insecticide was detected in the tracheal trunks and showed an increase with time of exposure. The haemolymph gave no detectable image.

On the basis of all these results, Gerolt concluded that contact insecticides accumulate in the integument, probably the endocuticle, and spread laterally to reach the site of action by way of the integument in the tracheal system.

In earlier experiments by Eoy and co-workers (1943) with cockroaches, paralysis was caused by the introduction of powdered pyrethrum into the tracheae via the spiracles. They considered that the toxicant reached the ganglia by diffusing through the tracheal walls into the haemolymph rather than following the direct route through the tracheal system.

Burt and co-workers (1971) conducted experiments which were designed to test Gerolt's hypothesis. In one study, three different methods of insecticide presentation were compared. Doses ( $\text{LD}_{95}$ ) of pyrethrin I were applied to cockroaches either, (a) in a solution of acetone on the metathoracic sternum or, (b) in a solvent-free emulsion with saline on the metathoracic sternum or, (c) in a solvent-free emulsion with saline into the tracheal system through a meta-

thoracic spiracle. The tracheal route of pyrethrin I entry was no more effective than the topical route and, in fact, appeared to be slightly less efficient over a 48 hour period.

In another experiments the sixth abdominal ganglion and the associated tracheal systems were exposed in isolated abdomens of cockroaches. Pyrethrin I was introduced into one of the longitudinal ventral trachea close to the ganglia in 0.15  $\mu$ l aliquots in a solvent-free emulsion. Only large doses of pyrethrin I produced any change in spontaneous nerve activity when introduced into the tracheae. Activity was greatly decreased but without the initial burst of activity observed when ganglia are irrigated externally with high concentrations of pyrethrin I.

Burt et al, (1971) have cast further doubts on Gerolt's theory on the basis that even after reaching the ends of the tracheoles within the CNS, an insecticide still faces a barrier of glial cells before reaching the neurones. They have suggested that if insecticides can traverse these, it should be possible for them to reach the haemolymph by diffusion through the tracheal walls.

Although the phenomenon of cuticular permeability is considered to be an essential occurrence in the model generally accepted for insecticide penetration, that is, passage through the cuticle into the haemolymph and thence to the CNS, Ebeling (1974) recognised the importance of three physico-chemical factors; liposolubility, surface migration and phase partition, which contribute to the

overall permeability of cuticle to insecticides. The active compound is usually presented to the cuticle as a solution in oil or in a more volatile organic solvent and, in many cases, the choice of solvent can be crucial. For example, Lewis (1963) has demonstrated that red flour beetles Tribolium castaneum absorb dieldrin from hydrocarbon oil solutions at rates which vary inversely with the viscosity of the oil and the size of the oil molecules. Lewis (1962) has also provided evidence of surface migration on insect cuticle by showing that when insects walk over fine deposits of oil, the whole of the external surface becomes covered within minutes with at least a monomolecular film.

Phase partition effects assume significance in cuticle permeability in that an insecticide must be able to migrate from the wax of the epicuticle to the underlying hydrophilic layers of the procuticle and beyond. It has been found that pyrethrin I, although much more liposoluble than diazoxon, is absorbed by the cockroach P. americana at a slower rate and this has been attributed to its lesser solubility in the aqueous phase within the insect (Burt and Lord, 1968; Burt et al., 1971).

#### 1.4. Aims of the work.

A dramatic escalation has been observed in recent years in the levels of human louse infestation in many countries and, as a result, much attention has been focussed on the need for new and effective insecticides. The development of improved insecticides for the control of lice is extremely important in view of the potential for the spread of disease associated with widespread infestations, and also the fact that insecticide resistance has been observed amongst head lice.

The colony of human body lice maintained at the London School of Hygiene and Tropical Medicine is one of perhaps, only three or four laboratory colonies in the world and provided the ideal opportunity to carry out several lines of research on this ectoparasite. Although the body louse has been the subject of numerous investigations much earlier in this century, interest was centred mainly on physiological, taxonomic and behavioural characteristics.

In an attempt to bring the information on this insect up to date and in line with what is currently known of other species, I have endeavoured to make use of as many advanced techniques as possible. The overall approach, however, has been to investigate those areas of research which might yield the most worthwhile data for use in formulating insecticidal preparations in general.

## 2. Materials and Methods.

### 2.1. Examination of the structure of body louse cuticle.

#### 2.1.1. Light microscope (LM) examination.

##### 2.1.1.1. Examination of unstained cuticle sections.

Male and female adult lice (2-3 days old) were snap-frozen by submerging in isopentane (melting-point - 159.9°C) cooled in liquid nitrogen. The lice were then embedded in Tissus - Tek II O.C.T. Compound (G.T. Gurr, Ltd., London, S.W.6) on separate cutting blocks and placed in the refrigerated compartment of a Slac cryostat (Slac Medical Equipment Ltd., London, S.E. 13) maintained at - 25°C. Sections ranging in thickness from 6 to 8  $\mu$  were cut from various locations on the lice and examined under oil using a Zeiss Photomicroscope II.

##### 2.1.1.2. Examination of stained cuticle sections.

Mature male and female adult lice, fed and unfed, were fixed separately in Deboscq Brasil (G.T. Gurr Ltd., London, S.W.6) for 48 hours at room temperature prior to dehydration in Supercedrol (G.T. Gurr Ltd.) for 72 hours with two changes of the solvent during this period. The fixed lice were placed in molten Paraplast (G.T. Gurr Ltd.) for 96 hours and then solidified in fresh Paraplast to form blocks for cutting. Sections (5-10  $\mu$ ) were cut from the blocks with a rotary microtome (A. Gallenkamp and Co. Ltd., London, E.C. 2P), and the most satisfactory results were obtained when the blocks had been previously chilled at 4°C for 1 hour.



Material produced in this way was stained with either Mallory's triple stain (G.T. Gurr Ltd.) or Hematoxylin and Phloxine (G.T. Gurr Ltd.) and examined as before (see section 2.1.1.1.).

2.1.2. Transmission electron microscope (TEM) examination.

2.1.2.1. Examination by TEM of cuticle sections.

Unfed male and female adult lice (2-3 days old) were cut into quarters under an ice-cold solution of 5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2. After fixation in the solution for 2 hours at 4°C, the specimens were washed with two changes of 0.1M cacodylate containing 0.5M sucrose for a further 20 minutes at the same temperature. Post-fixation was carried out in 1% osmium tetroxide in the cacodylate buffer at 4°C for 1 hour. The specimens were then washed as above at room temperature and dehydrated in an alcohol series before being subjected to two 5 minute washes in propylene oxide. Embedding took place in Araldite at 60°C over 12 hours after the specimens had been brought through a propylene oxide - Araldite series in which the Araldite concentration was progressively increased. Sections of cuticle approximately 50 nm and 100 nm thick were cut on an LKB ultramicrotome (Ultratome III - LKB Instruments Ltd., South Croydon, Surrey) and floated onto 400 - mesh, formvar coated copper grids and post stained with uranyl acetate and lead citrate. Examination of the sections under low and high resolution was

performed using transmission electron microscopes, Zeiss EM9, AEI 6B and AEI 801.

All chemicals and grids used throughout the procedure were obtained from EM scope Laboratories Ltd., London, S.W.8

2.1.2.2. Platinum/carbon (Pt/C) replication of outiocular surface and cross section.

Unfed male and female adult lice (2-3 days old) were dissected at the junction of the thorax and abdomen and as much of the contents as possible removed from the abdominal cuticle under distilled water. After a further wash in distilled water, specimens of abdominal cuticle were allowed to stand overnight in a solution of 20% glycerol in distilled water prior to freeze-fracturing. The impregnated specimens were positioned in metal stubs which were then placed into a small container of Freon 22 held in liquid nitrogen ( $-196^{\circ}\text{C}$ ). These rapidly frozen specimens and stubs were then installed in the vacuum chamber of a freeze-fracture unit (BAF 301 High Vacuum Freeze-Etch Unit, Balzers High Vacuum Ltd., Berkhamsted, Herts.) where temperature and pressure were reduced to  $-110^{\circ}\text{C}$  and  $2 \times 10^{-6}$  torr respectively. A mechanically operated razor was used to cut some frozen specimens while others were left intact. All specimens remained frozen and under vacuum during shadow-coating (at angle  $45^{\circ}$ ) with Pt/C then support-coating (at angle  $90^{\circ}$ ) with carbon. Replicas formed in this way were floated off in distilled water and recovered on TEM grids for examination in Zeiss EM9 and AEI 6B transmission electron microscopes.

2.1.3. Scanning electron microscope (SEM) examination.

2.1.3.1. Morphology and surface topography of the adult louse.

Whole, unfed male and female adult lice (2-3 days old) were fixed and post-fixed as described (see section 2.1.2.1.) and then critical point dried in acetone in a Polaron E3000 dryer (Polaron Equipment Ltd., Holywell Industrial Estate, Watford, Herts.) attached to a Churchill thermocirculator (Churchill Instrument Co. Ltd., Uxbridge, Middlesex). Individual specimens were mounted on SEM stubs and coated with a thin layer of gold in an Edwards S150 sputter coater (Edwards High Vacuum, Crawley, West Sussex) in preparation for examination at low resolution in a JEOL JSM-T20 scanning electron microscope (JEOL UK Ltd., Colindale, London N.W.9) and high resolution in a JEOL JEM - 100 CX TEMSCAN.

2.1.3.2. Examination of cuticular cross section.

Cryostat sections were prepared as described (see section 2.1.1.1.) using unfixed adults and fixed (see section 2.1.2.1.) critical point dried adult lice. The specimens were mounted and sputter coated prior to SEM examination of the cross sectional faces (see section 2.1.3.1.). Fractured specimens were prepared by gently tearing the carcasses of fixed, dried adults and the resultant fragments were then mounted on SEM stubs. Examination was carried out after sputter coating.

2.1.3.3. Solvent effects on the outicoular wax layer.

Whole, unfed adult lice (male and female, 2-3 days old) were agitated for 10 minutes in either acetone or hexane prior to fixation and critical point drying as described (see section 2.1.3.1.). Specimens were prepared and examined by SEM also as described (see section 2.1.3.1.).

2.1.3.4. Effects of alkali treatment on the cuticle.

Specimens of abdominal outicouls from unfed male and female adult lice (2 - 3 days old) were dissected as described (see section 2.1.2.2.) and submerged in 37% potassium hydroxide (KOH) at room temperature for 24 hours. The treated specimens were then dried in air and prepared and examined by SEM as described (see section 2.1.3.1.).

## 2.2. Chemical Composition of Louse Lipids.

### 2.2.1. Comparison of potential extraction solvents for cuticular lipids.

#### 2.2.1.1. Trial thin-layer chromatography (TLC) separations.

The following organic solvents were selected as potentially suitable for extraction of the cuticular lipids from the louse.

- i acetone
- ii benzene
- iii chloroform
- iv chloroform/methanol (2 : 1)
- v diethyl ether
- vi dimethoxymethane
- vii ethyl acetate
- viii hexane.

A 10ml aliquot of each solvent was placed into separate 25ml beakers followed by 200 adult lice (unsexed) and the mixtures gently agitated. After a 10 minute extraction, the contents of the beakers were filtered through separate small, sintered glass (size 3A) funnels and the extracts evaporated almost to dryness in a stream of nitrogen. Five microlitre samples of each extract were then spotted onto separate lanes of a prewashed TLC plate (20 cm x 20 cm Merck, Kieselgel HF 254 + 366, BDH Chemicals Ltd., Middlessex, U.K.) which was subjected to a 2-step, 1 dimensional development according to Skipski *et al* (1965). This comprised development first with isopropyl ether/acetic acid (96:4) until the solvent front ascended 13 cm., drying in nitrogen and development again in the same direction with petroleum ether (40° - 60°C)/

diethyl ether/acetic acid (90 : 10 : 1). All TLC solvents had been previously double-distilled in glass. On completion of the second development (solvent front ascended to 19 cm.), the plate was dried in nitrogen and placed in an atmosphere of iodine vapour. The resultant spots were noted and the plate then sprayed with 40% sulphuric acid before heating in an oven for 3 hours.

2.2.1.2. Solubility of long chain hydrocarbon ( $n - C_{36}$ ) in potential extraction solvents.

A 1 ml aliquot of each test solvent (numbers i to viii) was placed into separate stoppered 5 ml tubes. Five milligrams of hexatriacontane ( $n - C_{36}$ ) was added to each tube and the mixtures shaken. After 10 minutes at room temperature ( $25^{\circ}C$ ) the solvents in which the hexatriacontane had dissolved were noted.

2.2.2. Extraction of louse lipids.

2.2.2.1. Cuticular lipid extraction using whole lice.

Hexane proved the most satisfactory solvent for extraction of cuticular lipids (see section 3.2.1.) and was used exclusively for this purpose in all subsequent experimental work. Without prior purification however, commercially pure hexane was found unsuitable in preparing lipid extracts for eventual gas chromatographic analysis. Minor contaminants were concentrated along with the cuticular lipids during evaporation of the hexane in the nitrogen stream.

Hexane used throughout all experimental work was obtained as 'pure' hexane (Nanograde, Mallinckrodt) from Camlab Ltd., (Cambridge, U.K.) and then double-distilled using a spinning band column.

Pasteur pipettes (short form) were prepared as illustrated in Fig. 1 . Lice for solvent extraction were weighed collectively (e.g. 17.75 g, approx. 22,000 lice), then placed in the pipettes filling them to the three-quarters level. Rubber bulbs were connected to the pipettes (Fig. 1 ) and enough pure hexane to cover the lice drawn from a 25 ml reservoir of the solvent. To minimise contamination of the lipid extract, glassware used throughout all procedures had been previously cleaned in chromic acid and rinsed finally in acetone.

After 10 minutes, the hexane was expelled from the pipettes into the reservoir and the procedure repeated rapidly twice to rinse the contents. The combined solvent extracts were then evaporated to dryness in a nitrogen stream and the mass of remaining lipids determined using a Cahn Electrobalance.

On a number of occasions, adult lice were separated according to sex, counted and weighed prior to hexane extraction as above. After extraction, the lice were thoroughly dried and weighed.

#### 2.2.2.2. Cuticular lipid extraction using cast skins.

Cast skins were collected from large colonies of lice (in excess of 100,000 insects) over a period of one week. Removal of extraneous matter (e.g. faeces and dead bodies) was accomplished by placing the crude mixture in a vertical glass column

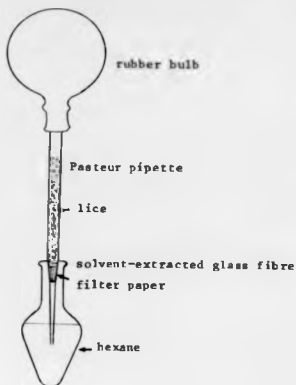


Figure 1. Extraction of cuticular lipids from whole lice.

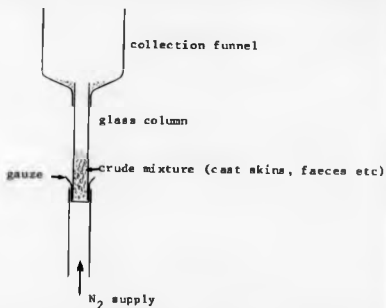


Figure 2. Collection of louse exuviae.



connected to a gentle stream of nitrogen entering at the base (Fig. 2 ). Being the lightest of the mixture, the skins were readily carried to the top of the column for collection. When enough skins had been collected, they were removed and washed in distilled water, dried and refluxed in chloroform for 6 hours in a Soxhlet apparatus. The chloroform extract was evaporated to dryness in a nitrogen stream and the recovered lipids dissolved in several microlitres of hexane in preparation for TLC analysis. The quantity of lipids extracted was minute and prevented accurate weighing. TLC analysis of the lipids produced spurious results (see section 3.2.3.1.) and all further analyses of cuticular lipids were performed on samples from whole insects.

#### 2.2.2.3. Examination of cuticular lipid extraction technique (using whole lice).

A colony of lice (8.68  $\pm$  g, approximately 10,000 lice) of both sexes and at various stages of development were collected for feeding on a live rabbit. The rabbit (adult female New Zealand white) was anaesthetized with an injection of Saffan (alphaxalone 0.9% (w/v), alphadolone acetate 0.3% (w/v); Glaxovet Ltd., Greenford, U.K.) and all fur removed from the stomach area with electric clippers. Blood (5.5 ml) was collected in a defibrinated syringe from vessels of both ears. Tritiated oleic acid ( $5 \text{ mCi}$ ;  $n_9$ ,  $10 \text{ H}^3$ ) (The Radiochemical Centre, Amersham, U.K.) dissolved in toluene was added to 5% (rabbit) albumin in 0.5 ml of Kreb's Ringer solution ( $\text{Ca}^{++}$  omitted)(see Appendix). The toluene solution, which floats

on the albumin solution, was evaporated in a gentle stream of nitrogen and 5 ml of the blood added with thorough mixing. A small volume (0.5 ml) was retained for scintillation counting. The labelled blood was re-injected into the rabbit via vessels in the ears and allowed to circulate for 10 minutes. Feeding of the lice colony was then permitted for 10 minutes upon the exposed skin of the rabbit's stomach. Blood samples were taken from shallow incisions in the area of lice feeding, immediately before and after feeding. The rabbit was sacrificed by injection immediately after removal of the lice. Extraction of the lice with hexane followed (see section 2.2.2.1.) and the dead lice retained. Lipids extracted by the hexane were recovered after evaporation of the solvent and weighed before adding to a vial containing 15 ml of scintillation solution (0.6% w/v PPO and 0.02 % w/v POPO in toluene). The dead lice were homogenised in a glass tissue grinder with a total volume of 50 ml. of distilled water and the homogenate sonicated (Rapidis sonicator; Ultrasonic Ltd., Shipley, Yorks. U.K.) for 5 minutes at 100 watts. A fine suspension was formed which was extracted three times for lipids using chloroform/methanol (2 : 1). These extracts were combined, filtered and the solvent evaporated to recover the lipids which were finally dissolved in scintillation solution as above. Blood samples were centrifuged at 3000 rpm using a Beckman TJ-6 benchtop centrifuge with TH4 rotor and 40µl aliquots of supernatant sera spotted and dried on separate scintillation

discs (Whatman 2.5 cm circles, Whatman Labsales Ltd., Maidstone, Kent) before adding to scintillation vials as above.

Scintillation counts were performed at 4°C (Packard 3003 scintillation spectrometer) for 1 minute periods for the following specimens:

- i 40 µl blood serum before radio-labelling
- ii 40 µl blood serum before lice feeding
- iii 40 µl blood serum after lice feeding
- iv extracted cuticular lipids
- v extracted whole body lipids
- vi blank (15 ml scintillation solution only - for background).

#### 2.2.2.4. Extraction of internal lipids from whole adult lice.

Twenty female adult lice (2 - 3 days old) were fed simultaneously with the large louse colony on radio-labelled rabbit blood (see section 2.2.2.3.). After 10 minutes, the lice were removed and held alive in an incubator for 24 hours (temperature 30°C and relative humidity 75%). The faecal matter was then removed by washing the insects in 10 ml of distilled water and the washings retained for lipid extraction (see section 2.2.2.5). Cuticular lipids were collected as described (see section 2.2.2.1.) and the insect bodies homogenised with 15 ml of distilled water, sonicated and extracted, also as described (see section 2.2.2.3.).

The procedure described above, was repeated using two larger colonies of lice consisting of 1000 males and 1000 female adults

respectively; however, feeding took place on unlabelled rabbit blood (i.e. separate rabbit). In both instances, lipid extracts were evaporated to dryness in a nitrogen stream and dissolved in approximately 5  $\mu$ l of hexane before TLC analysis (see section 2.2.3.).

#### 2.2.2.5. Extraction of lipids from louse faeces.

The faecal washings (10 ml) collected from the adult lice fed on radio-labelled rabbit blood (see section 2.2.2.4.) were sonicated and extracted for lipids as described (see section 2.2.2.3.). Extracted lipids were obtained by evaporating the solvent extract to dryness in a nitrogen stream, then prepared for TLC analysis (see section 2.2.3.) by dissolving in 5  $\mu$ l of hexane.

Faecal washings (20 ml each) were also collected from the two colonies of adult lice (1000 males and 1000 females respectively) described (see section 2.2.2.4.) and combined prior to sonication and lipid extraction as above. Recovery <sup>and</sup> of the extracted lipids from the solvent, <sub>and</sub> their preparation for TLC analysis also followed the above procedure.

#### 2.2.3. Thin-layer chromatographic (TLC) analysis of lipid extracts.

##### 2.2.3.1. Qualitative TLC analysis of cuticular lipids/internal lipids/faecal lipids.

TLC plates (20 cm x 20 cm) were prepared using Merck Kieselgel HF<sub>254</sub> + 366 spread in layers of 0.3 mm thickness. The plates

were dried, guide lines drawn and subsequently given a chromatographic run in chloroform as an effective means of washing. After drying and activation at  $110^{\circ}\text{C}$  for 1 hour, cleaned plates were spotted with 1  $\mu\text{l}$  aliquots of the following lipids in hexane:

- i cuticular lipids from unsexed whole lice (see section 2.2.2.1.)
- ii cuticular lipids from cast skins (see section 2.2.2.2.)
- iii internal lipids from adult male lice - fed on normal rabbit blood (see section 2.2.2.4.)
- iv internal lipids from adult female lice - fed on normal rabbit blood (see section 2.2.2.4.)
- v faecal lipids from adult (male and female) lice - fed on normal rabbit blood (see section 2.2.2.5.).

Lipid standards (see below) were dissolved in hexane and 1  $\mu\text{l}$  of each solution spotted on the plates prior to TLC development, thus enabling comparison with the unknowns to be made.

- |    |                            |   |                      |
|----|----------------------------|---|----------------------|
| a. | hydrocarbon                | - | hexatriacontane      |
| b. | cholesteryl ester          | - | cholesteryl stearate |
| c. | methyl ester               | - | methyl stearate      |
| d. | triglyceride               | - | triolein             |
| e. | free fatty acid            | - | oleic acid           |
| f. | 1, 3 - diglyceride         | - | 1, 3 - diolein       |
| g. | 1, 2 - diglyceride         | - | 1, 2 - diolein       |
| h. | cholesterol                | - | cholesterol          |
| i. | 1 - and 2 - monoglycerides | - | mono olein (90%)     |

The plates were developed under the same conditions as those described in 2.2.1.1. After completion of the TLC run the plates

were thoroughly dried under nitrogen and exposed to iodine vapour. When the spots became obvious, the plates were observed under UV radiation (366 nm) and the results recorded by photographing with a Polaroid CU-5 Land Camera (Polaroid Ltd., St. Albans, Herts. U.K.) fitted with a Wratten UV 2B filter (Kodak Ltd., Holborn, London). Detection with 40% sulphuric acid and heat was carried out and the resultant spots photographed under both natural light and UV radiation (366 nm).

#### 2.2.3.2. Quantitative TLC analysis (by weight) of cuticular lipids.

Preparative TLC plates (20 cm x 20 cm) were formed using Merck Kieselgel 60 PF <sup>254 + 366</sup> spread in 1.5 mm layers. Cuticular lipids from the extraction of approximately 15,000 unsegregated lice were spotted in a band formation along the origin and subjected to a one dimensional, two step development as described (see section 2.2.1.1.). After completion of the second development, the plates were dried in an atmosphere of nitrogen and exposed to iodine vapour to locate the lipid classes. Each lipid class was collected separately by carefully scraping the silica gel from the plates and placing it in Pasteur pipettes prepared with glass wool instead of filter paper as described (see section 2.2.1.1.). Lipids were eluted from each pipette with pure toluene and collected in separate 20 ml flasks. The toluene was then removed by evaporation in a stream of nitrogen and each lipid class dissolved in 20 µl of hexane to allow transference to small vials previously weighed on a Cahn Electrobalance. After evaporation of the solvent, the vials were reweighed to determine the lipid weights. Two

replicates were performed.

2.2.3.3. Quantitative TLC analysis (by densitometer) of  
cuticular lipids/internal lipids/faecal lipids.

The plates containing TLC separations of the following lipids  
(see section 2.2.3.1.) were used in the quantitative estimations;

- i cuticular lipids from unsexed whole lice
- ii internal lipids from adult male lice - fed on normal  
rabbit blood
- iii internal lipids from adult female lice - fed on normal  
rabbit blood
- iv faecal lipids from adult (male and female) lice - fed  
on normal rabbit blood.

After visualisation of the lipid classes with 40% sulphuric  
acid and heating, the plates were examined using a scanning  
densitometer (E.C. densitometer; Camlab Ltd., Cambridge, U.K.)  
to produce a linear representation of the TLC separations. Peaks  
from the same separations were then cut out and weighed and  
percent composition calculated from the weights.

2.2.3.4. TLC analysis of digestion products of labelled fatty  
acid (Oleic acid).

The twenty adult female lice fed on rabbit blood labelled  
with tritiated oleic acid (see section 2.2.2.3.) were extracted  
after 24 hours for cuticular and internal lipids (see section  
2.2.2.4.). Faecal matter expelled during this period was also  
extracted for lipids (see section 2.2.2.5.) and the total of  
each extract separated into lipid classes and recovered as

described (see section 2.2.3.2.). The various lipid classes were counted for radioactivity as described (see section 2.2.2.3.) over 10 minute periods.

#### 2.2.3.5. Test for saturation of cuticular hydrocarbons.

Cuticular hydrocarbons were examined for saturation using the method of Jackson et al (1974). TLC plates were prepared as described (see section 2.2.3.1.) and sprayed with 95% ethanol (in water) saturated with silver nitrate. The plates were then heated to 100°C for several minutes to remove the ethanol and allowed to cool. Those not used immediately were stored in darkness in a desiccator. Cuticular hydrocarbons and reference standards were spotted onto plates for development in hexane followed by visualisation with 40% sulphuric acid spray and charring with heat.

#### 2.2.4. Gas-liquid chromatographic (GLC) analysis of lipid extracts.

##### 2.2.4.1. Modification of Perkin-Elmer F11 gas chromatograph (GC) for capillary column use.

Initially, GC equipment consisted of a single channel flame ionisation detector (FID) gas chromatograph (Perkin-Elmer F11) coupled to a temperature programmer. The instrument was not specifically designed for use with glass columns; however, glass columns were prepared with dimensions allowing exact alignment with the existing inlet and outlet column couplings inside the GC oven.



Two columns (10ft x 3/32 inch and 5 ft x 3/32 inch) were packed with 7% OV 101 (Chrompack U.K. Ltd., London SW16) on Chromosorb W-HP (80 - 100 mesh) (Chrompack U.K. Ltd.) and 10% butanediol succinate (EBS) (Chrompack U.K. Ltd.,) on Chromosorb W-HP (80 - 100 mesh) respectively.

An homologous series of straight chain alkanes (carbon numbers 15 to 25) were eluted from both columns with temperature programming from 70°C to 190°C and 270°C for EBS and OV101 phases respectively, separation of alkanes containing more carbons was found increasingly difficult and higher final oven temperatures appeared necessary. Methyl silicone, OV101, proved a more suitable stationary phase than EBS under these conditions because of its higher stability at the increased temperatures (maximum temperature limit: OV101 (350°C), EBS (200°C) ).

The small quantities of lipids available for analyses proved a limiting factor in the use of the packed glass columns. Injection of volumes less than 1 µl required operation of the FID amplifier at maximum sensitivity; however, baseline increase under these conditions, due to phase bleed and FID sensitivity change with temperature, was excessive. In response to the problems encountered, particularly that of using minute samples, it was decided to install a capillary column in the GC.

Fig. 3 shows the final arrangement with the capillary (10m x 0.3mmWCOT OV101 - Phase separations Ltd., Clwyd, U.K.) attached to the FID Analyser Unit. The restricted space within

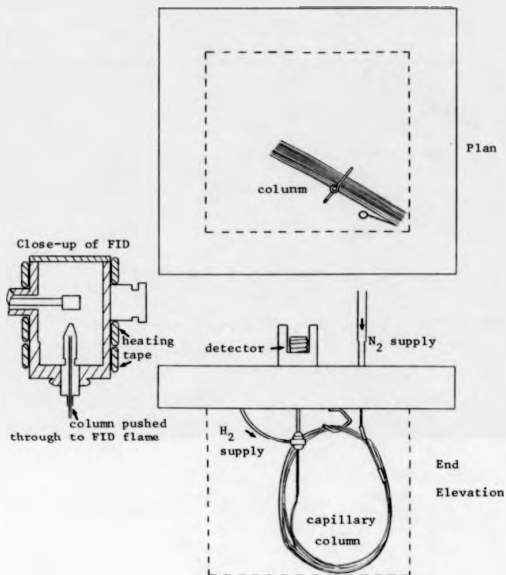


Figure 3. Installation of capillary column in Perkin-Elmer F11 GLC.

the small GC oven governed the placement of the column (coiled diameter 11 cm.). It was suspended diagonally in the front half of the oven, avoiding all obstacles. All gas connections to the column were made with pre-shrunk PTFE tubing (internal diameter 0.5 mm.) capable of enduring temperature up to 260°C without deformation. Prior to connection of the PTFE tubing, it was important for the sharp glass edges of the capillary column to be gently abraded with fine emery paper and the ends of the PTFE tubing opened out with a pencil point.

The injection system of the F11 was unsuitable for use with the capillary column and, instead, nitrogen carrier gas was supplied directly to the column via PTFE tubing attached to nickel tubing through the insulated oven lid. A straight length of glass capillary tubing (approximately 5 cm.) was inserted between the nickel supply line and the column to facilitate introduction of samples for analysis. The carrier gas supply was controlled by a toggle valve positioned close to the column and was switched off while the straight glass tubing was gently removed from the system. Samples for analysis were deposited on the internal surface by capillary action, ensuring that excessive volumes of solvent were not ultimately introduced into the column itself, leading to removal of the stationary phase. This was avoided by allowing most of the solvent to evaporate before replacing the straight tubing in the system. After re-connection, the gas supply was snapped on simultaneously with the temperature programmer which was set from room temperature to 260°C. The carrier gas

pressure ( 8 psi) was pre-set for the system to give a flow rate of 25 cm. sec<sup>-1</sup> for methane. Stability and sensitivity of the FID were optimised by employing two modifications. Firstly, the FID was insulated from the main oven and maintained at a steady, controllable temperature between 300°C and 350°C using a length of insulated heating cord coiled around the exterior of the detector (Fig. 3 ). Secondly, the flame-drawn end of a straight length of glass capillary tubing was inserted through the metal tubing connected to the FID to stop a few millimetres short of the FID flame (Fig.3 ). The swagelock connection towards the other end was fitted with a graphite ferrule to secure the capillary tubing in place and provide a gas-tight seal. With the hydrogen supply also connected at this junction, an effective sweep of the volume preceeding the FID was established. Using the system described above, it was possible to obtain excellent chromatograms from sample volumes less than 1 µl.

#### 2.2.4.2. Initial GLC separation of cuticular hydrocarbons.

Cuticular lipid samples from segregated male and female adult lice (see section 2.2.2.4.) were prepared by TLC as described (see section 2.2.3.2.) and subjected to GLC analysis with the system described (see section 2.2.4.1.). The column used for the GLC separations was an OV 101 WCOT glass capillary column (10 mm x 0.3 mm) temperature programmed from room temperature to 270°C at 10°C minute.<sup>-1</sup> Nitrogen (oxygen-free) was the carrier gas used at a pressure of 8psi at the column inlet. As comparison showed the chromatograms to be identical, further analyses

were conducted using cuticular hydrocarbons from unsexed adults for quantitative purposes. Peak areas in subsequent chromatograms were estimated using an electronic integrator (Venture Digital Integrator Mk II; Smiths Industries, London SW2.) and in turn, percent composition of the component hydrocarbons was calculated.

#### 2.2.4.3. GLC examination for saturation of cuticular hydrocarbons.

Cuticular hydrocarbons from unsexed adult lice were separated according to saturation on a silver nitrate impregnated TLC plate (see section 2.2.3.5.). The regions of silica gel containing the saturated and unsaturated hydrocarbons were located under UV radiation (366 nm), scraped from the plate and the hydrocarbons eluted separately from the material with pure hexane (see section 2.2.3.2.). Both solutions were evaporated to dryness in a stream of nitrogen and redissolved in 10  $\mu$ l of hexane prior to GLC analysis under the conditions described (see section 2.2.4.2.).

Unsaturated hydrocarbons were removed from a second cuticular hydrocarbons sample using bromination. A solution of bromine in toluene was added to the hydrocarbon sample also dissolved in toluene and allowed to stand overnight at  $-10^{\circ}\text{C}$ . The brominated hydrocarbons were recovered after evaporation of the toluene and bromine and analysed under the GLC conditions described (see section 2.2.4.2.). Chromatograms produced by brominated and unbrominated cuticular hydrocarbons were then compared and the differences noted.

2.2.4.4. GLC examination for branched/unbranched cuticular hydrocarbons.

A cuticular hydrocarbon sample was added to 10 ml of 2,2,4 - trimethylpentane containing activated Linde molecular sieve 5A as 1/16 inch pellets. The mixture was allowed to stand for 24 hours and then filtered (O'Connor et al, 1962). Normal hydrocarbons were taken up by the molecular sieve while the branched hydrocarbons remained in solution and were recovered after evaporation of the solvent. GLC analysis under conditions as described (see section 2.2.4.2.) identified the chromatogram peaks produced by branched hydrocarbons.

2.2.4.5. Comparison of unknown GLC peaks with standard GLC peaks.

An homologous series of normal hydrocarbons (carbon numbers 15 to 32) was prepared and chromatographed as described (see section 2.2.4.2.). Comparison of the retention times produced by the homologous series with those of the cuticular hydrocarbons indicated the carbon chain lengths of the unknowns. The hydrocarbon series was then co-chromatographed with the cuticular hydrocarbons and the resulting chromatogram closely examined for coinciding peaks which indicated hydrocarbons of equivalent carbon number.

2.2.4.6. Position of double bond in unsaturated cuticular hydrocarbons.

Unsaturated cuticular hydrocarbons (see section 2.2.4.3.) were subjected to a periodate - permanganate oxidation (Downing and Greene, 1968) which caused cleavage at the double bonds. From the products

of the cleavage, it was possible to deduce the structure of the major alkenes.

Briefly, 5 mg of unsaturated cuticular hydrocarbons was dissolved in 10 ml. of pure *t*-butyl alcohol and added to 10 ml. of distilled water containing 120 mg of sodium periodate and 10 mg of potassium permanganate. The solution was brought to pH 8-9 with the addition of aqueous potassium carbonate, stirred for four days then extracted with methylene chloride to remove neutral lipids. After neutralisation with aqueous sodium bisulphite, the remaining aqueous solution was extracted with chloroform which was dried, filtered and evaporated to dryness. The fatty acids which were formed by the reactions, were methylated by dissolving in 2% sulphuric acid in anhydrous methanol and heating to 70°C in a closed tube. After 1 hour, the methyl esters formed were recovered by ether extraction and analysed by GLC as described (see section 2.2.4.2.).

2.2.4.7. Gas chromatography/Mass spectroscopy (GC/MS) analysis of cuticular hydrocarbons.

Cuticular hydrocarbons from unsexed adult lice were analysed by GC/MS using both Electron Ionisation (E.I.) and Chemical Ionisation (C.I.).

The first analyses were performed using E.I. on a Kratos MS 25 mass spectrometer coupled to a Data General DS-50 computer system. The glass capillary column (10 m x 0.3 mm OV101 WCOT)

used in previous GLC analyses (see Materials and Methods) section 2.2.4.2.) was installed in the GC unit (Sigma 3) and connected directly to the spectrometer. Helium at 5 psi (flow, 2 ml minute<sup>-1</sup>) was used as the carrier gas and the GC oven programmed from 100°C to 260°C at 10°C minute<sup>-1</sup> during the GC run. Injection (splitless) temperature was 300°C while the spectrometer inlet was held at 275°C. The spectrometer was operated with Electron Energy at 70 eV, Ionising Current 100 uA, Accelerating Voltage 1.33 KV and source temperature at 200°C.

An analysis was performed with the system described above using the mass spectrometer in the C.I. mode and ultrapure methane as the ionising gas. Operating conditions were duplicated with the exception of Electron Energy which increased to 100 eV and Ionising Current became Emission Current 5 mA.

The cuticular hydrocarbons were also examined using a Finnigan 1020 GC/MS system. A 50 m glass WCOT SE 30 capillary column was used for the GC separation with temperature programming from 150°C to 270°C at 10°C minute<sup>-1</sup>. Mass spectral data were processed by a computer incorporated in the system.

#### 2.2.4.8. GLC analysis of internal hydrocarbons/faecal hydrocarbons.

Internal lipids from 1000 male and 1000 female adult lice respectively were collected (see section 2.2.2.4.) and the hydrocarbons obtained using column chromatography with alumina



(Merck, neutral, activity grade 1; BDH Chemicals Ltd., Middx. U.K.) and hexane as the elutant.

Faecal lipids were extracted from the combined faecal washings of the colonies described above (see section 2.2.2.5.). The hydrocarbons were separated and collected as above and the three extracts concentrated by evaporation in a stream of nitrogen. GLC analyses were performed using a Perkin-Elmer Sigma 5 gas chromatograph fitted with a 2m x 3mm packed (3% OV - 1 on Chromosorb W-HP, 100-120 mesh) glass column and temperature programming from 150°C to 300°C at 10°C minute<sup>-1</sup>. Injection and detector blocks were heated to 350°C and nitrogen (oxygen-free) flowing at 32 cm<sup>3</sup> minute<sup>-1</sup> was the carrier gas. n-Alkanes were identified by comparison of retention times and co-chromatography with authentic standards.

Faecal hydrocarbons were also analysed by GC/MS using a Kratos MS25 mass spectrometer. The preliminary GC/MS separation was performed on a 50 m OV 101 WCOT glass capillary column with temperature programming from 150°C to 300°C at 10°C minute<sup>-1</sup>. All other operating conditions were identical to those described (see section 2.2.4.3.).

#### 2.2.4.9. GLC analysis of cuticular cholesteryl esters.

Cuticular lipids were separated into lipid classes by TLC and each collected (see section 2.2.3.2.). A 0.5 mg sample of cholesteryl esters was added to 5 ml. of 2% sulphuric acid in

anhydrous methanol in a glass tube. The mixture was sealed and heated to 70°C for 1 hour then extracted with ether to recover the methyl esters produced. After evaporation of the ether, the esters were redissolved in 5 µl of hexane and analysed using a Pye Unicam Series 204 gas chromatograph fitted with a 10m x 0.3 mm OV101 WCOT glass capillary column. An injection splitter (S.G.E. Pty. Ltd., London NW2) was used at the injection end of the column while the straight end at the detector stopped several millimetres short of the FID flame as described (see section 2.2.4.1.) to enhance sensitivity. Capillary column connections were PTFE tubing as described (see section 2.2.4.1.) which limited the upper temperature to 260 - 270°C. Nitrogen (oxygen-free) carrier gas was supplied at 7-8 psi and the GC separation temperature programmed from 100°C to 265°C at 10°C minute<sup>-1</sup>. Comparison of retention times and co-chromatography with standards assisted in identifying the main methyl/ester peaks, while electronic integration (Venture Digital Integrator MK II; Smiths Industries, London NW2) was used to calculate their percent composition.

#### 2.2.4.10. GLC analysis of cuticular triglycerides.

The triglyceride fraction from a TLC separation of cuticular lipids (see section 2.2.3.2.) was treated in the same manner as the cholesteryl esters (see section 2.2.4.9.) converting each triglyceride fatty acid component into a fatty acid methyl ester (F.A.M.E.). GLC analysis of the methyl esters was performed on the Pye Unicam Series 204 gas chromatograph under conditions as described (see section 2.2.4.9.). The saturation of the methyl esters was not investigated.

2.2.4.11. GLC analysis of cuticular fatty acids.

The fatty acid fraction was collected from a TLC separation of cuticular lipids (see section 2.2.3.2.) and methylated as described (see section 2.2.4.9.). Conditions for GLC analysis of the fatty acid methyl esters were as described (see section 2.2.4.9.) using the same equipment. No examination of fatty acid methyl ester saturation was carried out.

2.2.4.12. GLC analysis of cuticular 1, 3-diglycerides.

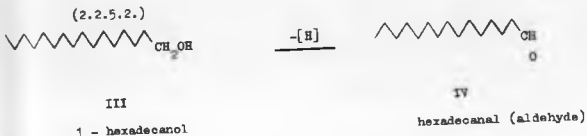
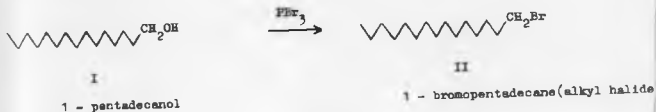
The 1, 3-diglyceride fraction was collected from a TLC separation of cuticular lipids (see section 2.2.3.2.) and treated with 2% sulphuric acid in anhydrous methanol as described (see section 2.2.4.9.) to form fatty acid methyl esters. GLC analysis of the methyl esters was performed with equipment and conditions as described (see section 2.2.4.9.).

No examination of methyl ester saturation was carried out.

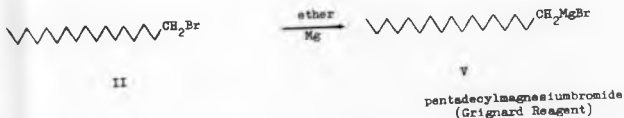
2.2.5. Synthesis of major unsaturated cuticular hydrocarbon  
(15 - hentriacontene).

The cuticular hydrocarbon 15 - hentriacontene, was prepared  
via the route indicated below.

(2.2.5.1.)



(2.2.5.3.)



(2.2.5.4.)

IV + V



VI Grignard intermediate

VI

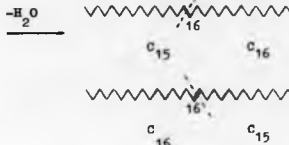


VII

16-hentriacontanol (secondary alcohol)

(2.2.5.5.)

VII



VIII

15 - hentriacontene (alkene)

#### 2.2.5.1. Preparation of alkyl halide (II) -intermediate .

1 - Pentadecyl bromide was prepared in a substitution reaction where the halogen replaced the hydroxyl group of 1 - pentadecanol (Noller and Dinmore, 1943).

An excess of phosphorus tribromide (5 ml.) was added with stirring to a solution of anhydrous diethyl ether containing 25 g of anhydrous 1-pentadecanol (I) while at  $-10^{\circ}\text{C}$ . The temperature of the mixture was kept below  $0^{\circ}\text{C}$  during the addition and was allowed to reach room temperature overnight. Crude halide was obtained by distillation of the mixture ( $160^{\circ}\text{C}$  at 10 mm pressure), washed twice with 5 ml. of concentrated sulphuric acid and shaken with dry potassium carbonate until the hydrogen bromide odour had disappeared. The halide was redistilled at atmospheric pressure (approximate temperature  $320^{\circ}\text{C}$ ) and the distillate (II) recrystallised from chloroform and weighed (16.65 g). Overall yield for the 1-pentadecyl bromide was 52.7% and the melting point found to be  $20.2^{\circ}\text{C}$ .

#### 2.2.5.2. Preparation of aldehyde (IV) - intermediate .

The primary alcohol, 1-hexadecanol, was oxidised to form the aldehyde (IV), 1-hexadecanal, following the method of Partch (1964).

A solution containing 15 g. of 1-hexadecanol in 150 ml. of pyridine was stirred while an equal quantity of powdered lead tetraacetate was added at room temperature. The solution turned

yellow after several hours indicating that exhaustion of the oxidant had occurred. Addition of lead tetraacetate was repeated as above until the solution finally remained red. The reduced lead tetraacetate was precipitated on cooling and removed by filtration, and after evaporation in vacuo of the pyridine, the crude hexadecanal was distilled at  $200^{\circ}\text{C}$  (30 mm pressure). TLC examination of the aldehyde (see section 2.2.3.1.) revealed the presence of hexadecanol as a minor impurity which was removed by column chromatography of the distillate on a silica gel column (1 cm x 60 cm). Hexadecanal was eluted first using hexane/diethyl ether (90:10 V/V) as the eluant and after recovery by evaporation of the solvent, its weight (8.26 g) represented a 57.2% yield. The melting point of the hexadecanal (IV) was  $32.5^{\circ}\text{C}$ .

#### 2.2.5.3. Preparation of Grignard Reagent (V) - intermediate.

A spherical reaction flask containing 2 g of pure magnesium turnings covered with 30 ml of anhydrous diethyl ether was fitted with two Quickfit dropping funnels, which effectively sealed the flask. Enough halide from a solution containing 16.65 g of 1-bromopentadecane in 20 ml of anhydrous diethyl ether was added from one dropping funnel to initiate the reaction. Addition of the solution was maintained at a slow rate until exhaustion to avoid violent boiling and the final mixture warmed slightly to ensure completion of the reaction. The freshly made Grignard Reagent was used immediately.

#### 2.2.5.4. Formation of secondary alcohol (VII) - intermediates.

A solution containing 8.26 g of hexadecanal (IV) in 20 ml of anhydrous diethyl ether was added dropwise to the Grignard Reagent (V) via the second dropping funnel of the apparatus as described (see section 2.2.5.3.). After addition of the whole aldehyde solution, 10 ml of ice-cold aqueous chloride solution was added to the mixture and the ether and aqueous layers allowed to separate before extracting the latter three times with diethyl ether. The united volumes of ether extracts were dried over anhydrous sodium sulphate and the crude alcohol recovered by evaporation of the ether and distillation at atmospheric pressure (approximate temperature  $390^{\circ}\text{C}$ ). Recrystallisation of the distillate (VII) from hexane produced 6.64 g of 16-hentriacontanol which represented a combined yield of 43.2% for procedures 2.2.5.3. and 2.2.5.4. A melting point of  $87^{\circ}\text{C}$  was recorded for the product.

#### 2.2.5.5. Formation of alkene (VIII) from alcohol (VII).

The secondary alcohol, 16-hentriacontanol, (see section 2.2.5.4.) was dehydrated using orthophosphoric acid to form the alkene (VIII), 15-hentriacontene following the method of Corson and Ipatieff (1955). One double bond per molecule was formed, which, in this case was possible on either side of the central carbon atom (carbon no. 16). The final products, however, were identical since both possible molecular structures consisted of 15 and 16 carbon chains on either side of the double bond



respectively (see diagram in section 2.2.5.). It was expected that both *cis* - and *trans* - forms would be produced in the reaction.

A distillation flask containing 6.64 g of 16 - hentriacontanol in 10 ml of 85% orthophosphoric acid was gradually heated at atmospheric pressure and water distilled off. Crude alkene was collected around 380°C and purified by column chromatography with alumina (Merck, neutral, activity grade 1) and hexane as the eluant. The product (VIII) weighed 3.59 g which represented a yield of 55.1% for the reaction and a melting point of 46°C recorded.

A GLC examination of the 15 - hentriacontene was carried out using a Perkin-Elmer Sigma 3 gas chromatograph under conditions as described (see section 2.2.4.8.) while a GC/MS examination was also performed using a Kratos MS25 mass spectrometer operating under conditions as described (see section 2.2.4.8.).

## 2.5. Applied aspects

### 2.5.1. Maintenance of lice colonies

All lice used in experiments throughout these studies were obtained from four large colonies which were originally established in 1972 using eggs from rabbit-adapted lice (Orlando strain) supplied by the U.S. Department of Agriculture in Gainesville, Florida.

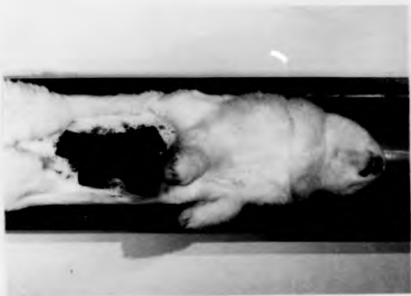
After feeding once daily, each colony of lice was placed in a separate glass beaker (diameter 15 cm) containing 3 cm squares of black corduroy fabric and kept in the dark in an incubator at 30° C and 75% relative humidity. Eggs were laid on the fabric squares in the colonies and removed with the material after two week periods when clean material was introduced. In general, eggs laid on the squares required 9 days for hatching and all nymphs had emerged by the eleventh day.

A group of 12 rabbits, mostly New Zealand white, were kept exclusively for the maintenance of the lice colonies. Preparation of the rabbits involved anaesthetisation with an intramuscular injection of Saffan (0.9% w/v alphaxalone, 0.3% w/v alphadolone; Glaxovet Ltd., Greenford, U.K.), then the stomach area shaved as closely as possible with electric clippers (Oster

Professional model A - 5) and loose fur removed with a vacuum-cleaner. The animal was then transferred to a plastic trough in which it lay on its back supported by the structure (Plate 1a). Nymphal feeding was commenced by placing the egg-laden fabric squares on the shaved stomach of the rabbit for 5 minutes allowing newly emerged nymphs to migrate from the squares. All squares were then removed and returned to the incubator while the nymphs continued feeding for a further 5 minutes until being collected for addition later to the main colonies.

Feeding of the main colonies followed a similar format whereby fabric squares and lice from a colony were placed on a rabbit for 5 minutes before returning the squares to the original container. At this point, the lice colony was cleaned of cast skins, faeces and other accumulated debris by gently vacuuming over the feeding lice while they remained securely attached to the skin of the rabbit. Time of feeding totalled 15 minutes which ensured that virtually all lice were fully engorged before their collection by hand from the surface of the rabbit. The small numbers of lice which escaped collection in this manner were removed by wiping the shaved stomach of the rabbit with cotton wool and destroyed. In practice, the populations of the colonies were allowed to reach 15,000 - 20,000 adults before reducing the numbers to around 5,000 adults per colony.

Plates 1a and 1b. Lice feeding on anaesthetised  
rabbit supported in trough.



2.3.2. Physical characteristics of cuticular lipids and adult lice.

2.3.2.1. Melting-point determination of cuticular wax.

Approximately 5000 unsexed adult lice were extracted for cuticular lipids as described (see section 2.2.2.1.) and the wax deposited in a melting-point tube. The melting-point temperature was determined using a Gallenkamp melting-point apparatus (A. Gallenkamp and Co. Ltd., London EC2P), programmed for a slow temperature increase. A total of 3 extractions were carried out and melting-point temperature determined for each extract.

2.3.2.2. Solubility of simulated cuticular wax in potential insecticide solvents.

A simulated wax mixture was prepared containing n - alkanes and n - alkene (see Table 1 ) in the same proportions as the corresponding naturally occurring compounds determined in section 3.2.4.2. Homogeneity was achieved by agitation of the molten mixture (60°C). The n - alkene (15 - hentriacontene) was prepared as described (see section 2.2.5.).

2 ml. vials were set up, each containing 1 mg of the simulated wax mixture. Ten potential insecticide solvents listed below

Table 1

Composition of synthetic cuticular wax





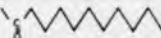





Hydrocarbon	Carbon No.	% Composition	Mass mg
n - tricosane	23	3.0	0.66
n - tetracosane	24	1.3	0.30
n - pentacosane	25	20.8	4.63
n - hexacosane	26	1.5	0.33
n - heptacosane	27	22.5	5.00
n - octacosane	28	2.5	0.56
n - nonacosane	29	23.3	5.19
n - triacontane	30	0.7	0.16
n - hentriacontane	31	1.5	0.33
15-hentriacontene	31	22.9	5.10

(Table 2 ) were then added in 100  $\mu$ l aliquots to the separate numbered vials and stoppered before placing in an incubator at 30°C and 75% relative humidity.

After 24 hours, the solvents in which the wax mixture had dissolved were recorded. Where the wax mixture had not dissolved, 100  $\mu$ l aliquots of the corresponding solvents were added to the vials which were then returned to the incubator for a further 24 hours period. The addition of solvents in 100  $\mu$ l increments was repeated until each 1 mg sample of wax mixture had dissolved and the volume of solvent necessary for dissolution in each case recorded.



Table 2. Potential insecticide solvents.

Compound	Melting-point (°C)	Empirical formula	Structural formula
1,4-butanediol	20	$C_4 H_{10} O_2$	
didecyl ether	16.5	$C_{20} H_{42} O$	
dodecanal	13	$C_{12} H_{24} O$	
dodecanol	22	$C_{12} H_{26} O$	
2-dodecanone	17	$C_{12} H_{24} O$	
lauryl ether	3-5	$C_{24} H_{50} O$	
1-octadecene	17.5	$C_{18} H_{36}$	
n-pentadecane	10	$C_{15} H_{32}$	
1, 2-propanediol	-60	$C_3 H_8 O_2$	
undecanol	19	$C_{11} H_{24} O$	

2.3.2.3. Determination of upper lethal temperature of adult lice.

Ten male and 10 female adult lice were collected 2-3 days after moulting (see section 2.3.3.1.) and carefully placed into separate melting-point tubes. Each louse was subjected to a slow increase in temperature (around  $1.66^{\circ}\text{C min}^{-1}$ ) in a Gallenkamp melting-point apparatus and the temperature at which the insect ceased all movement recorded.

In a second series of experiments, lice were placed in melting-point tubes and set up in turn with an electrode in contact with the louse as illustrated (Fig. 4 ). The electrode was a simple construction, consisting of two wires insulated from each other by means of a glass capillary tube and was connected in series to a 1.5 volt battery and pen recorder (Fig. 4 ).

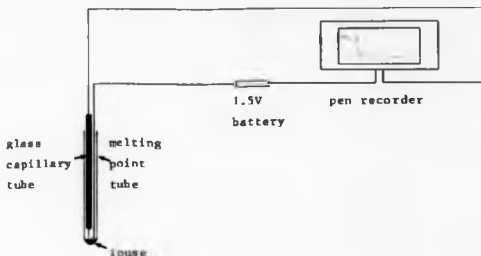


Figure 4. System for determination of upper lethal temperature.

As the temperature of the melting-point tube was slowly elevated using the melting-point apparatus, the recorder measured the potential of the circuit and recorded its variation with time and temperature.

#### 2.3.2.4. Comparative volatilities of potential insecticide solvents.

A solution was prepared containing 3 mg of simulated wax mixture in 250 ml. of hexane. Using a syringe, 1 ml aliquots of the solution were carefully deposited upon the horizontal surface of 10 clean microscope slides (26 mm x 75 mm) so as to cover the entire uppermost surface of the slides without spilling over the edges. Evaporation was allowed to take place at room temperature (22°C) resulting in the formation of a wax layer (approximately  $0.63 \text{ g cm}^{-2}$ ) with an average thickness of 0.66 micron (Looney, 1960).

Each of the potential insecticide solvents described earlier (Table 2) were then deposited as single 5  $\mu$ l droplets on the prepared microscope slides. The slides were held in an incubator at 30°C and 75% relative humidity and examined twice daily to establish the time taken for each solvent to completely evaporate in order to calculate rates of evaporation.

### 2.3.3. Toxicity tests with adult lice.

#### 2.3.3.1. Preparation of test material.

A degree of standardisation among the test insects was achieved by selecting only females which had moulted 2-3 days earlier and using them in the tests approximately 6 hours after last feeding. Initially, 250 nymphs at the pre-adult stage were collected from the main colonies while under anaesthetization in a gentle flow of carbon dioxide (Fig. 5) and returned separately to the incubator (see section 2.3.1.).

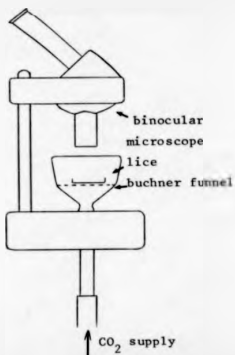


Figure 5. Equipment for collection of nymphs and adult female lice.

Following each 24 hour period, successive batches of 250 nymphs were set up to form a total of 4 batches whilst all adult males were removed from preceding batches. The lice were fed daily as described (see section 2.3.1) in conjunction with the main colonies.

At any time, the most mature sub-colony of lice contained in excess of 120 adult females, 2-3 days old for use in tests.

#### 2.3.3.2. Protocol for toxicity tests.

Toxicity tests followed a format whereby 120 adult female lice (100 test, 20 control), 2-3 days old (see section 2.3.3.1.) were placed in separate small containers. One hundred of these lice were topically applied with the potential insecticide solvent under a microscope.

In standardising the procedure, 3 types of containers (Plate 2) were used to hold the separate lice in a series of preliminary toxicity tests. One type of container (1 cm diam x 0.5 cm height) was composed of glass, and produced from out down vials while the other two were plastic and normally used as caps for bottles (Plastic A - Packard scintillation vial cap, 1.5 cm ID x 0.5 cm height, Plastic B - glass vial cap, 1.7 cm ID x 0.7 cm height). All containers were prepared for use in the tests by soaking for several hours (overnight) in a 3% solution of Decon 90 (Decon Laboratories Ltd.,



Plate 2. The 3 types of containers used to hold separate lice during preliminary toxicity tests. (Details in the text)

Portalade, Brighton, U.K.), rinsed twice with distilled water and dried while inverted at 60°C. The preliminary toxicity tests involved 4 potential insecticide solvents (dodecanol, 2-dodecanone, lauryl ether and undecanol) in conjunction with the 3 types of containers. Substantial differences were noted between the mortality rates obtained with the various containers (see section 3.3.2.1.) and consequently, the glass containers were chosen for use throughout all subsequent tests on the basis of their relative inertness in contact with the solvents.

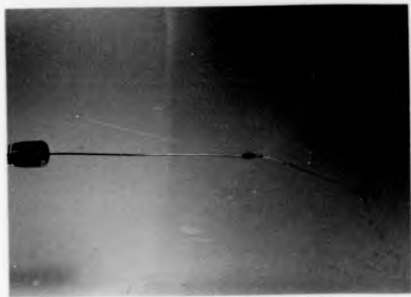
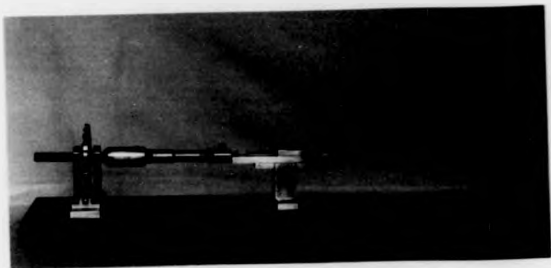
A topical applicator (after Busvine) (Plate 3) was used in the procedure and consisted of a 10  $\mu$ l microsyringe (SGE (UK) Ltd., London NW2 7AT) coupled to a micrometer. Capillary glass tubing, 4 cm in length and flame-drawn to 0.1 mm internal diameter, was attached with Araldite to the end of the steel syringe needle to facilitate application of 0.01 - 0.2  $\mu$ l volumes.

A relationship between the actual mass of solvent deposited by topical application and the corresponding number of micrometer divisions was established. At various dosage levels for the solvent, 20 droplets of equal volume were deposited collectively in pre-weighed glass containers produced by sealing one end of thin-walled glass tubing (internal diameter 1 mm) and cutting approximately 2 mm from the sealed end. Solvent and container were weighed on a Cahn Electrobalance and the mass of the 20 droplets found after subtraction of the container

Plate 3a. Topical applicator for toxicity tests of solvents upon adult lice. (After Busvine).

Plate 3b. Close-up of topical applicator showing fine glass tubing secured to syringe needle with Araldite.





weight. A graph was constructed for the solvent plotting mass of the topically applied droplet against the number of micrometer divisions. Volume of topically applied solvent was calculated using the following equation.

$$d = \frac{m}{v}$$

d - density of solvent

m - mass of droplet

v - volume of droplet

After topical application, treated and control groups of lice were placed in an incubator at 30°C and 75% relative humidity for 24 hours and then examined individually on the skin of the wrist under a microscope. Those lice which failed to take blood from the skin were classed as dead and mortality rates were calculated using these results.

Complete test results were analysed by computer (CDC 7600 computer) using a Genstat package to fit data with a probit model (designed for bioassays) by using regression characteristics. Probit-log graph paper was used to plot dosage (mass/lice on logarithmic axis) versus corresponding mortality rate (percent mortality on probit axis) for the solvent under test and the computer analysis provided information to construct a regression line through the points to give the lowest possible  $\chi^2$  value (i.e. goodness of fit). The Genstat programme also estimated the  $LD_{50}$  and  $LD_{90}$  (i.e. dosage causing 50% and 90% mortality respectively) with associated fiducial limits, slope of the

regression line with standard error and  $\chi^2$  value with number of degrees of freedom to obtain the P value. Significance was taken at the 5% level, which meant that dosage-mortality data resulting in P values lower than 0.05 were considered unreliable.

#### 2.3.3.3. Topical application of potential insecticide solvents.

The 10 selected solvents (see Table 2 ) were topically applied in the pure form and at four different dosage levels ( $\mu\text{g/louse}$ ) for each solvent to give mortalities ranging from the low percentages (20%-30%) to almost 100%. Three replicate tests were performed with each chosen dose of solvent. Lauryl ether and dodecanol were supplied by Rees and Carnrick Pharmaceuticals of Kenilworth, New Jersey, U.S.A. for testing while the remaining solvents were obtained from Koch-Light Laboratories Ltd., of Colnbrook, Bucks, U.K.

#### 2.3.3.4. Topical application of aqueous insecticide solvent mixture.

The most toxic potential insecticide solvent, lauryl ether (see section 3.3.2.2.) was thoroughly mixed with distilled water in the ratio 1:9 (V/V) to form a 10% homogeneous suspension. Toxicity tests were performed with the suspension as described (see section 2.3.3.2.) except that a 50  $\mu\text{l}$  Hamilton glass syringe

Table 3

Dosage levels for topical application  
of 10% aqueous lauryl ether mixture.

Number of micro- meter divisions	Estimated volume of 10% lauryl ether mixture ( $\mu$ l)
5	0.0416
10	0.0833
15	0.1250

It was not possible to accurately weigh droplets  
of the test mixture due to the rapid evaporation  
of the distilled water.

(Phase Separation Ltd., Queensferry, Clwyd, U.K.) fitted with a glass needle was used in the topical applicator to supply a larger volume range.

Three replicate tests were performed at each of the following dosage levels (Table 3) using the criteria for death as described (see section 2.3.3.2.) after the 24 hour incubation period.

2.3.3.5. Topical application of insecticide (dieldrin) in potential insecticide solvent.

Using the most toxic and the least toxic potential insecticide solvents, lauryl ether and 1, 4-butanediol respectively (see section 3.3.2.2.) solutions were prepared containing 0.04 g of dieldrin per ml (Shell Research Ltd., Sittingbourne, Kent, U.K.) and 0.004 g of dieldrin per ml. Topical application of these 4 solutions at a constant dosage volume of 0.005  $\mu$ l (i.e. 2.5 micrometer divisions) gave calculated amounts of dieldrin as shown in the following table.

Three replicate tests were performed with both solvents at either dieldrin concentration and mortality rates determined as described (see section 2.3.3.2.).

Table 4

Dosage levels for topical application of  
dieldrin in potential insecticide solvents

Insecticide solvent	Applied volume per louse	Dieldrin per louse (at conc <sup>n</sup> 40mg ml <sup>-1</sup> )	Dieldrin per louse (at conc <sup>n</sup> 4mg ml <sup>-1</sup> )
lauryl ether	0.005 ml	0.2 µg	0.02 µg
1,4-butanediol	0.005 ml	0.2 µg	0.02 µg

Table 4

Dosage levels for topical application of  
dieldrin in potential insecticide solvents

Insecticide solvent	Applied volume per louse	Dieldrin per louse (at conc <sup>n</sup> 40mg ml <sup>-1</sup> )	Dieldrin per louse (at conc <sup>n</sup> 4mg ml <sup>-1</sup> )
lauryl ether	0.005 ml	0.2 µg	0.02 µg
1,4-butanediol	0.005 ml	0.2 µg	0.02 µg

2.3.4. Observation of cuticular penetration by  
potential insecticide solvents using  
fluorescent dye (Rhodamine B).

Solutions were prepared with the most toxic potential insecticide solvents (lauryl ether and dodecanol) and the least toxic potential insecticide solvent (1, 4-butanediol), each containing 0.5% (w/v) Rhodamine B (G.T. Carr Ltd., London S.W.6.). Each solution was then used in the topical application of three separate batches of 10 female adult lice, 2-3 days old (see section 2.3.3.1.) respectively at a dose rate of 0.011  $\mu$ l per louse. Control insects were set up in an identical manner without the presence of Rhodamine B in the insecticide solvents. After 30 minutes in an incubator at 30°C and 75% relative humidity, three lice from each batch were washed in a stream of acetone and snap-frozen in isopentane cooled in liquid nitrogen in preparation for cutting cryostat sections as described (see section 2.1.1.1.).

At 1 and 4 hours, dead lice were removed from the batches of lice treated with lauryl ether and dodecanol respectively, as well as 3 lice each from the untreated and 1,4-butanediol treated batches and cryostat sections produced as above. The sections of control material, Rhodamine B treated material and the untreated material were examined using a Leitz Dialux



microscope fitted with Rhodamine filter system (UV-blue incident illumination, 610 nm barrier filter) (E. Leitz, Luton, Beds., U.K.). Exposure for photomicrographs was set automatically with a Wild exposure unit coupled to the fluorescent microscope.

### 3. Results.

#### 3.1. Examination of the structure of body louse cuticle.

##### 3.1.1. Light microscope (LM) examination.

##### 3.1.1.1. Examination of unstained cuticle sections.

During these studies, two distinct types of cuticle were identified at various locations on the body of the louse. Smooth, sclerotized cuticle (Plates 4 and 5) covered the head, thorax, appendages and paratergal plates whilst the abdominal integument was found to be rugose and membranous (Plate 6). Except for regions of modification where the integument was somewhat thicker such as various points on thorax and legs, cuticle thickness was essentially uniform throughout (approximately  $7\text{ }\mu$ ) irrespective of type. Tanning in the exocuticle and epicuticle of sclerites was marked by the appearance of a yellow or brown colouration (Plate 4) which occurred even more extensively in the thicker integument (Plate 5). Minimal tanning was observed in the membranous integument and was restricted to the region at the peaks of rugosities (Plate 6).

Pore canals were readily distinguished as striations in sclerotised cuticle, particularly in areas of greater thickness, and followed relatively direct routes between the epidermis and epicuticle. In sections of membranous cuticle however, pore canals appeared to be discontinuous and much less ordered than those described above which suggested that they followed rather circuitous paths through the procuticle.

Plate 4. Unstained cryostat section of sclerite cuticle with pore canals (arrows) appearing as continuous striations from epidermal layer (ed) to exocuticle(ex). Note the tanning in the epicuticle (ep) and exocuticle. Trapped air in some pore canals has caused them to appear darkened. en, endocuticle. x 2300.

Plate 5. Unstained cryostat section of dorsal thoracic cuticle showing extensive tanning and numerous pore canals following relatively direct paths. ep, epicuticle; ex, exocuticle; en, endocuticle. x 2300.



Plate 6. Unstained cryostat section of dorsal abdominal cuticle (membranous) with pore canals (arrows) following indirect routes through the endocuticle (en) resulting in discrete holes rather than continuous striations ep, epicuticle; ex, exocuticle; ed, epidermis. x 2300.



### 3.1.1.2. Examination of stained cuticle sections.

Longitudinal whole body sections stained with the nuclear staining dyes Hematoxylin/Phloxine were particularly informative, revealing the locations within the body of the important structures (Plate 7). The head capsule and thorax of both sexes were occupied primarily by musculature which was associated either with movement of mouthparts and antennae in the case of the head or of legs in the case of the thorax. The alimentary canal and reproductive organs were the dominant internal features in the abdomen, with the former becoming considerably distended during feeding to fully occupy the anterior half of this section of the body.

An examination of transverse body sections stained with Mallory's triple stain (Plates 8 and 9) confirmed these anatomical observations and provided histochemical information on the types of cuticle present. In addition to the usual areas of head, thorax, legs and paratergites, sclerotised cuticle was also located in distinct strips across abdominal segments. These dorsal and ventral sclerites, known as tergites and sternites respectively (Plates 8 and 9), were present in adults of both sexes but were more extensive in males. In Plate 10 (paratergite cuticle), the exocuticle and epicuticle were distinguished by their colouration due to tanning whilst the endocuticle, traversed by pore canals, remained unstained.

Un-sclerotised, membranous cuticle readily accepted Mallory's triple stain which accentuated the laminated character of the

Plate 7. Whole body section of (fed) female adult louse stained with Hematoxylin/Phloxine. The advanced development of the eggs (ovaricles) gives some indication of the maturity of the insect.

- 1 cibarial muscles
- 2 lateral pharyngeal muscle
- 3 antennal muscles
- 4 ocular lobe
- 5 brain
- 6 spiracle
- 7 dorsal lateral muscle
- 8 vertical muscle
- 9 notal pit
- 10 salivary glands
- 11 stomach (mid-gut)
- 12 ovariole
- 13 oviduct
- 14 uterus
- 15 accessory reproductive glands
- 16 pylorus
- 17 vagina
- 18 gonopod



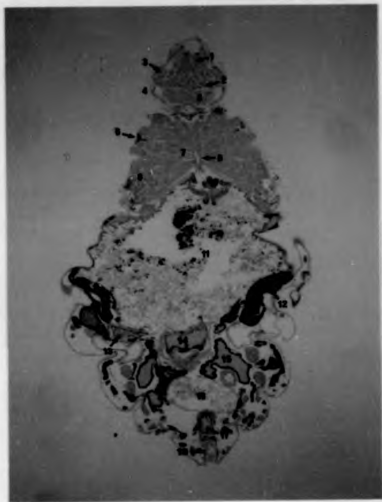


Plate 8. Transverse section through abdomen of unfed female adult louse. Note the sclerotised cuticle (t, tergite) and ovarioles (ov) at various stages of development. s, seta; sp, spiracle; m, musculature; g, gut. (Mallory's triple stain) x 100.

Plate 9. Transverse section through abdomen (anterior) of engorged female louse. Membranous cuticle has expanded whilst the sclerotised cuticle (st, sternite) remains rigid. bm, blood-meal; d vm, dorsoventral muscle; p p, paratergal plate; lm, lateral muscle. (Mallory's triple stain) x 100.

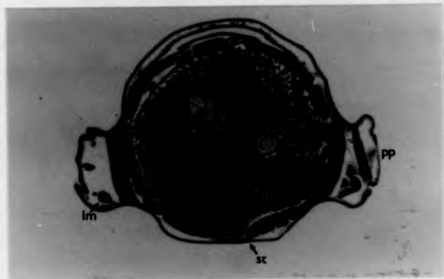
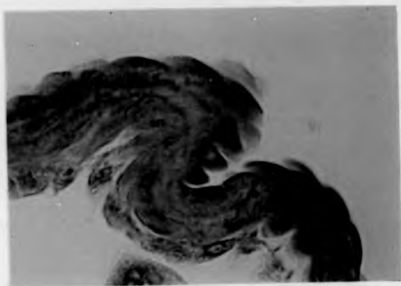


Plate 10. Sclerotised outicle from an unfed male adult louse. Tanning is evident in epicuticle (ep) and exocuticle (ex) layers. The endocuticle is traversed by numerous pore canals but remains unstained by Mallory's triple stain. ed, epidermis. x 100.

Plate 11. Unsclerotised outicle from abdomen of an unfed male adult louse. After staining with Mallory's triple stain, the exocuticle (ex, rendered orange in the photomicrograph) is separated from new endocuticle (en, blue) by an intermediate layer containing both colours.



structure. The red staining exocuticle (appearing orange-red in Plate 11) was separated from the blue-staining endocuticle by a mauve layer (mesocuticle) apparently containing both colours and denoting an intermediate region. Epicuticular tanning, if present, could not be distinguished because of the general level of staining which also camouflaged the pore canals.

### 3.1.2. Transmission electron microscope (TEM) examination.

#### 3.1.2.1. Examination by TEM of cuticle sections.

Cuticle sections showed the multilaminate construction characteristic of arthropods in general. The sequence of layers in both types of cuticle, namely sclerite and membranous, was identical with a thin epicuticle outermost, overlying a relatively thicker exocuticle and endocuticle respectively, with an epidermal layer at the base.

#### The epicuticle

At least four layers were identified within the epicuticle of sclerite and membranous cuticle and the combined thickness of the layers varied between 0.1 and 0.2  $\mu$ . An electron-dense membrane with an average thickness of 30  $\text{\AA}$  lay outermost and followed strictly the contours of the cuticle surface. A 50  $\text{\AA}$  thick layer, the outer epicuticle, separated the outer membrane from the cuticulin and was completely electron-lucent

(Plates 13 and 14). In the epicuticle of sclerite cuticle, the cuticulin was rather ill-defined and, although extremely electron-dense at the boundary with the outer epicuticle, it became less so on approaching the underlying dense homogeneous layer (Plate 14). However, the average thickness of the cuticulin in both cuticle types was estimated to be approximately  $100 \text{ \AA}$  whilst the dense layer formed the remainder of the inner epicuticle thickness. Differentiation between dense layer and underlying exocuticle was more marked in the membranous cuticle (Plate 17) than in sclerite cuticle (Plate 13).

In some instances where the cuticle surface had been relatively protected during embedding in Araldite, electron-dense deposits were observed in areas adjacent to the surface (Plates 13 and 16) and were probably the remnants of the surface wax layer or the cement layer or both.

A series of tubules running parallel to the surface of the cuticle was located at the interface of the epicuticle and pro-cuticle (Plate 17). In vertical sections the tubular network was quite distinct from the epicuticular filaments and appeared as a single row of electron-lucent holes which, in most instances, possessed electron-dense circumferences. Diameters ranged between  $250$  and  $400 \text{ \AA}$  (Plate 17).

### The exocuticle

The exocuticle thickness in sclerites of adults varied between 1.0 and 1.4  $\mu$ , being composed of two distinct layers of approximately equal thickness (Plate 15). Both regions possessed a granular appearance; however, the innermost was the less electron-dense of the two and was, in fact, the least electron-dense of all cuticle layers. The outer region of the sclerite exocuticle was dominated by a profusion of electron-dense filaments extending in both horizontal and vertical directions. In passing from the outer to the inner exocuticle, successive attachment among filaments caused a noticeable reduction in numbers until only single pore canals remained (Plate 15).

In the rugose, membranous cuticle, the exocuticle occupied a rather irregular volume bounded loosely by the epicuticle and the base of each rugosity (Plate 18). As with the sclerite exocuticle, maximum thickness of the layer averaged 1.0 to 1.4  $\mu$  and was also divided into two different regions (Plate 17). Both regions contained unidirectional, closely-packed macrofibrils arranged parallel to the longitudinal axis of each rugosity or corrugation. Macrofibrils in the outer region (average thickness 0.5  $\mu$ ) were of medium electron-density and clearly defined by more dense outlines which aided the measurement of macrofibril diameters (250 - 300  $\text{\AA}$ ). This layer followed closely the contours of the cuticle surface whilst the underlying inner exocuticle occupied the remaining space above the outer endocuticle. Macrofibrils in the inner exocuticle were somewhat larger in diameter (i.e. 400 - 1000  $\text{\AA}$ ) and less densely packed than those described above (Plate 17).



### The endocuticle

In sclerite cuticle, the endocuticle (3.0 - 4.5  $\mu$  thick) possessed a reasonably homogeneous granular appearance but did not exhibit the lamellate structure commonly encountered in arthropod cuticle (Plates 15 and 19). Individual chitin crystallites were not resolved in the micrographs, but the uniform appearance of the endocuticle was consistent with that of unidirectionally oriented crystallites frequently observed in the endocuticle layer of many other arthropods.

The endocuticle of membranous cuticle however, consisted of a well-defined lamellate structure (Plates 16, 17 and 18) formed by the helicoidal arrangement of 250-1000  $\text{\AA}$  diameter macrofibrils. Outer lamellae were generally much wider than those more recently deposited but all were composed of relatively few macrofibril layers which suggested that the angles between successive macrofibril planes were large. Deposition by the underlying epidermal layer often resulted in the lamellae being thrown into quite convoluted patterns (Plates 17 and 18) but the basic helicoidal arrangement was still preserved. The fusion of sclerite (tergite) with membranous cuticle shown in Plate 18 illustrated the gradual transition from the unidirectionally oriented chitin crystallites in the former to the macrofibrils with helicoidal arrangement in the latter. Endocuticle structure in membranous cuticle was also noted to be less closely-packed than in sclerite cuticle.

### The epidermis

The epidermis underlying both sclerite and membranous cuticle consisted of a single layer of epidermal cells and was limited basally by the basement lamella. Dimensions of the epidermal layer were dependant upon developmental state and the location on the louse body such as a smooth layer under sclerite cuticle or a rugose layer under membranous cuticle; but the average thickness lay between 0.5 and 3.0  $\mu$  (Plates 15, 16, 18 and 19). In sclerite integument the apical cellular surface of the epidermis appeared to be separated from the procuticle by a granular layer and a plasma membrane respectively (Plate 19) though this was not so readily apparent in membranous integument because of its convoluted nature (Plate 18). Each epidermal cell contained a large nucleus with a prominent nucleolus whilst the remaining cytoplasm consisted primarily of densely-packed free ribosomes and rough and smooth endoplasmic reticulum in addition to numerous mitochondria (Plates 15, 18 and 19). Desmosomes were observed forming the lateral attachments between adjacent cells as well as hemidesmosomes performing a similar function between cells and the basement lamella (Plate 19).

### The pore canal system

Pore canals with diameters from 0.1 to 0.2  $\mu$  were observed traversing the cuticle from the apical epidermal surface to the inner exocuticle (Plates 15, 18 and 19). Earlier observations which indicated that the paths of the pore canals through sclerite

endocuticle were more direct in comparison to those through membranous endocuticle (see section 3.1.1.) were confirmed conclusively at the ultrastructural level (Plates 15 and 16). In both types of integument the half of the pore canals proximal to the epidermis appeared as electron-lucent structures bounded by a cytoplasmic membrane approximately 100 Å thick and containing one or more filaments of about 200 Å diameter (Plates 15, 18 and 19). It was noted that the filaments entered the epidermal layer (Plate 19) and this was particularly evident in integument from immature adults (Plate 16). It could not be ascertained whether or not they actually entered the epidermal cells.

The distal half of the pore canals showed heavy staining by osmium tetroxide, including both the cytoplasmic membrane and the internal contents (Plates 15 and 18). Some minor branching was observed in the outer endocuticle but most occurred in the exocuticle and with such frequency that the system in this layer was more filamentous than canal-like (Plates 15, 17 and 18). The exocuticle of solerites contained significantly greater numbers of filaments than that of membranous cuticle (Plates 15 and 17) and this disparity also existed in the epicuticular layers of both types of cuticle. Below the layer containing the tubular network, the filaments followed both vertical and lateral routes through the exocuticle; however, all the epicuticular filaments above the tubular network traversed the epicuticle normal to the surface (Plates 14 and 17). Each epicuticular filament was

Plate 12. Paratergal plate (pu) on abdomen of female adult louse (2-5 days old) showing junction of sclerite cuticle with membranous cuticle. ~~Arrow~~ indicates location of cuticle in Plate 13. x 350.

Plate 13. Sclerite cuticle from area shown in Plate 12. Deposits (d) of wax and/or cement layer are seen in a state of gradual removal adjacent to the cuticle surface. sp, spiculate; ex, exocuticle. x 32000.

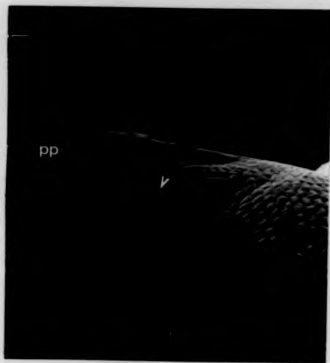


Plate 14. Sclerite cuticle from abdomen of male adult louse (2-3 days old). Epicuticular filaments (ef) can be seen connected with the surface via pores. om, outer membrane; oe, outer epicuticle; c, cuticulin; dl, dense layer; ex, exocuticle. x 126000.

Plate 15. Sclerite cuticle from abdomen of female adult louse (2-3 days old). The homogeneous granular endocuticle (en) appears to be non lamellate and is traversed by pore canals (pc) passing directly through the matrix. oex, outer exocuticle; iex, inner exocuticle; en, endocuticle; apm, apical plasma membrane; ed, epidermis; n, nucleus; bm, basement lamella. x 10800.

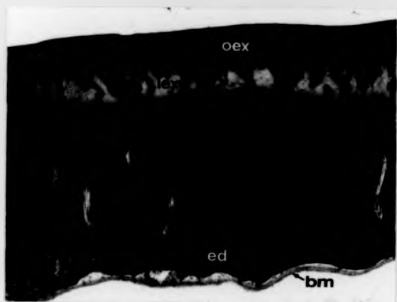


Plate 16. Membranous outicle from the abdomen of post-ecdysial female adult. The exocuticle is large in comparison with the endocuticle (composed of only 3 lamellae). Pore canals appear to penetrate the epidermis. ep, epicuticle; ex, exocuticle; en, endocuticle; ed, epidermis; bm, basement lamella; m, muscle. x 1500.

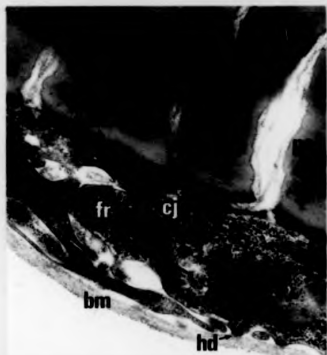
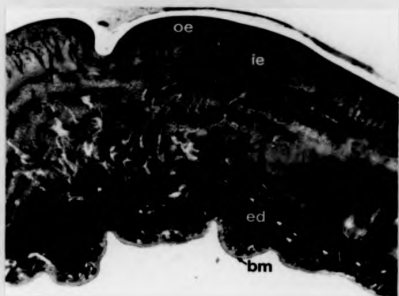
Plate 17. Membranous outicle from abdomen of female adult (2-3 days). The tubular network is visible underlying the epicuticle and the macrofibrillar nature of the exocuticle/endocuticle layers is also evident. ep, epicuticle; tn, tubular network; oe, outer exocuticle; ie, inner exocuticle; en, endocuticle; pc, pore canal. x 23000.





Plate 18. Junction of sclerite/membranous cuticle on abdomen of male adult louse (2-3 days) showing fusion of helicoidal macrofibrils in membranous endocuticle with unidirectional microfibrils in sclerite endocuticle. ce, outer exocuticle; i e inner exocuticle; pc, pore canal; en, endocuticle; ed, epidermis; bm, basement lamella. x 8400.

Plate 19. Sclerite abdominal integument from female adult louse (2-3 days) showing cuticle deposition zone adjacent to apical plasma membrane of epidermis. pc, pore canal; apm, apical plasma membrane; fr, free ribosomes (and rough/smooth EPR); cj, cell junction; bm, basement lamella; m, mitochondrion; hd, hemidesmosome. x 42000.



about 100 Å in diameter and linked with the surface of the cuticle via a small depression or pore which was approximately 100 Å at its base (Plate 14).

3.1.2.2. Platinum/Carbon (Pt/C) replication of cuticular surface and cross section.

The cuticular surface was found to have a covering of fairly evenly spaced small mounds, each approximately 0.1 µ in diameter at the base (Plate 20). On membranous cuticle, the mounds occurred at the rate of 80-90 per square micron which was consistent with the occurrence of the epicuticular filaments in the corresponding TEM sections (i.e. 8-10 epicuticular filaments per micron). A similar situation was observed for sclerite cuticle except the mounds were found to be approximately three times as numerous which resulted in a more uniform appearance. That the mounds were part of the surface wax bloom was demonstrated by the fact that whole sections of the layer were stripped from the cuticular surface during freeze-fracturing (Plate 21).

Examination of oblique cross sections of membranous integument (Plate 21) confirmed that the macrofibrils of the exocuticle were oriented unidirectionally along the length of the rugosities. In the underlying endocuticle, the macrofibrils appeared to form a lamellate structure which was consistent with the observations made during the TEM studies (see section 3.1.1.1.). The angular differences in orientation between two successive layers of macrofibril

Plate 20. Surface replica of membranous abdominal outcicle from female adult louse (2-3 days) demonstrating the wax bloom formed by extruded cuticular wax. x 22000.

Plate 21. Replica of surface and oblique cross section of membranous outcicle from male adult louse. Cuticular wax has been stripped away in sheets to reveal a smooth surface. Pore canals appear as holes (arrows) throughout the endocuticle. es, outcicle surface; ex, exocuticle; en, endocuticle. x 4000.

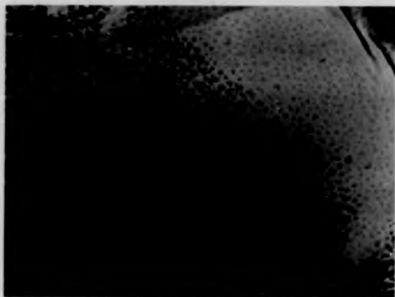
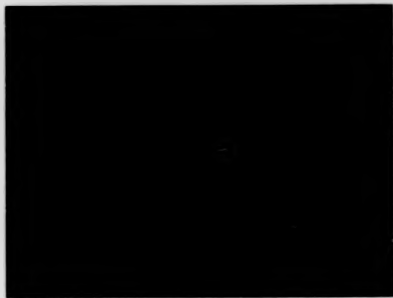


Plate 22. Replica of an oblique cross section through endocuticle of membranous cuticle from female adult louse (2-3 days). Two adjacent planes of macrofibrils can be seen (arrows) separated by approximately  $45^\circ$ . x 40 000.

Plate 23. Replica of oblique cross section through endocuticle/epidermis region of membranous cuticle from female adult louse (2-3 days). Circular holes indicate pore canals following vertical routes while elongated holes or channels result from canals following lateral routes. en, endocuticle; ed, epidermis. x 10 000.





planes in the endocuticle was about  $45^{\circ}$  (Plate 22). Many pore canals, particularly in the outer half of the endocuticle, were cut obliquely during the fracturing procedure and produced elongated holes (Plate 21) whilst those produced by pore canals in the endocuticle adjacent to the epidermis were irregular (Plate 23).

### 3.1.3. Scanning electron microscope (SEM) examination.

#### 3.1.3.1. Morphology and surface topography of the adult louse.

The anterior extremity of the head was formed by a membranous protuberance (haustellus) surrounding the mouth opening (Plate 26) through which the internal mouthparts were retracted when not feeding. Two large areas of sclerite integument (clypeus and ocular-antennal segment) separated by arthrodial integument (clypeofrontal suture) completely covered the dorsal surface of the head and were extended ventrally to some degree (Plate 26). A single, well-developed eye was located on the ocular lobe posterior to each antenna (Plate 26) which, in turn contained five segments. Peg organs or sensilla basiconica (Torre-Buneo, 1962) were observed on the extremity of the fifth segment of each antenna whilst tuft organs or sensilla coeloconica (Torre-Buneo, 1962) were restricted to the fourth and fifth segments (Plate 26). Tactile hairs were observed on each antennal segment (Plate 26) and in fact, occurred extensively on other parts of the body.

The dorsal surface of the thorax displayed only slight segmentation as a result of the fusion of the pleurites or subcoxae (Plates 24 and 27). Whilst the metathorax had almost entirely disappeared, the notum was reduced to a narrow, membranous axial strip encompassing the notal pit. The one pair of spiracles present on the thorax were located on the mesothoracic subcoxae and were totally isolated by sclerotised cuticle (Plates 25 and 27). Ventrally, the thorax consisted essentially of an enlarged, sclerotised sternal plate which showed no apparent segmentation. Legs were highly modified crab-like structures especially adapted for clasping hairs and fibres etc. Each leg possessed a tibia with extended thumb coupled to a single-jointed tarsus bearing a tarsal claw (Plate 25).

Some reduction in abdominal segments was observed at the anterior and posterior ends of the adult louse which resulted in only 7 segments being readily apparent (plate 24). The membranous integument which covered most of the abdomen was of a rugose nature and existed in two forms (Plates 27, 28 and 29). One type (Plate 28) consisted of long corrugations whilst the other (Plate 29) seemed to be merely an attenuated version of the first and exhibited a 'quilted' appearance. Sclerite cuticle on the abdomen was restricted to a series of plates (paratergal plates) at the lateral margins of each of the first 6 segments (Plates 24 and 25) as well as much smaller strips on the dorsal and ventral surfaces of each segment. Each paratergal plate bore a single

Plate 24. Female adult louse, 2-3 days old. On the abdomen two series of pits, parallel to the paratergal plates, can be seen. Some areas containing serrated rugosities are indicated by arrows. th, thorax; n, notum; np, notal pit; ab, abdomen (segmented); p, pits; pp, paratergal plate. x 35.

Plate 25. Female adult louse, 2-3 days old. an, antenna; ps/mes/mts, prothoracic/mesothoracic/metathoracic subcoxae; c, coxa; cl, claw; ta, tarsus; ti, tibia; sp, spiracle. x 35.

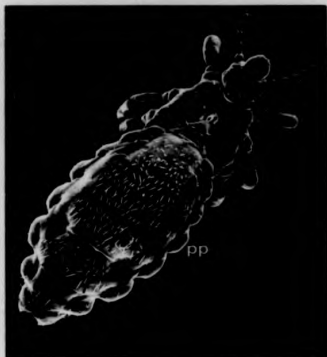


Plate 26. Close-up of female adult louse (Plate 24). The mouthparts have been retracted into the head. h, haustellum; l, labrum; c, clypeus, cs, clypeo-frontal suture; th, tactile hair; sb, sensillum basiconica; sc, sensillum coeloconica; as, antennal segment; e, eye; ol, ocular lobe. x 100.

Plate 27. Thorax of male adult louse (2-3 days) showing close fusion of the three segments. The notal pit is surrounded by membranous outcils of the notum, itself almost entirely surrounded by sclerite outcils. n, notum; np, notal pit; sp, spiracle; ap, abdominal pits. x 100.

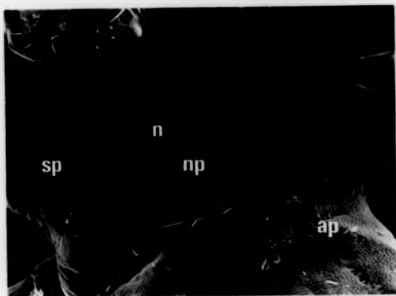
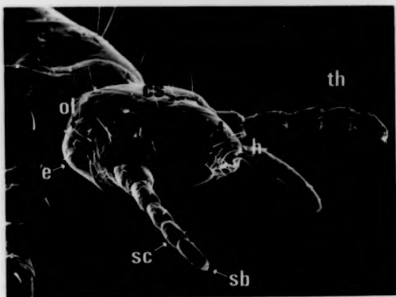


Plate 28. Membranous abdominal cuticle from male adult louse (2-3 days). Distinct patches occur in which rugosities possess serrations on their posterior edges and are found on the dorsal and ventral surfaces of each segment. x 600.

Plate 29. Membranous abdominal cuticle from female adult louse (2-3 days) showing individual pits which form the series found on dorsal and ventral surfaces of both sexes. x 630.

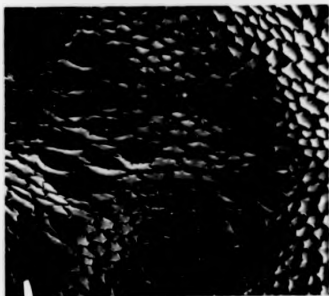
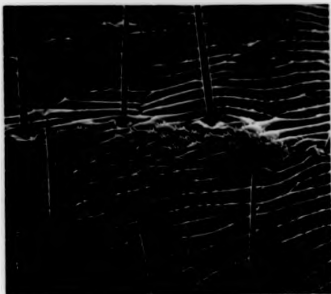




Plate 30. Membranous cuticle from abdomen of male adult louse, 2-3 days old. The surface wax layer has been severely disrupted as a result of the SEM preparation procedure and the wax bloom is not evident. x 3500.

Plate 31. Sclerite cuticle from thorax of female adult louse (2-3 days) showing the distribution of the surface wax which has occurred as a result of preparation for SEM examination. x 2000.

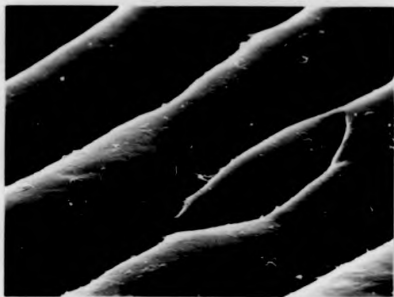
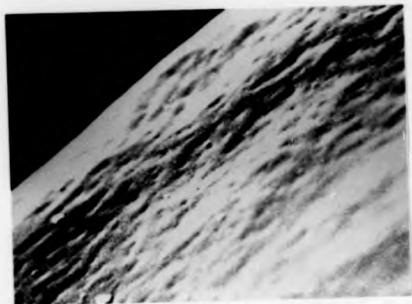


Plate 32. Membranous cuticle from abdomen of male adult louse, 2-3 days old. Surface wax has been dissolved during the SEM preparation procedure and deposited at a later stage as an irregular film. x 60 000.

Plate 33. Sclerite cuticle from thorax of female adult louse (2-3 days) on which surface pores (arrows) can be seen through the irregular wax film. x 60 000.



Plate 34. Solerite outcicle from paratergal plate of female adult louse, 2-3 days old. The surface wax layer has been completely dissolved and removed during preparation for SEM examination revealing the surface pores. x 50 000.



spiracle (Plate 25) displaying the distinctive mushroom shape characteristic of Pediculus. Dorsal and ventral segments of the abdomen possessed two elongated regions where the posterior edges of the rugosities were serrated (Plate 28) and pits (Plate 29) were also observed along the outer boundaries of the segments towards the paratergal plates forming two continuous series on both abdominal surfaces (Plates 24, 25 and 27).

The cuticular wax layer on both membranous (Plates 30 and 32) and sclerite (Plates 31 and 33) integument experienced severe disruption during the SEM preparation procedure despite initial osmium fixation. Unlike the regular appearance exhibited by the extruded wax mounds in platinum/carbon replicas (Plate 20), the cuticular wax was spread over the surfaces as an even film (Plates 31, 32 and 33) as a result of dissolution during critical point drying. On close examination of sclerite surfaces, there appeared to be epicuticular pores concealed beneath the dispersed wax film (Plate 33) and were more clearly seen in areas where the wax had been completely removed (Plate 34).

#### 3.1.3.2. Examination of cuticle cross section.

Unfixed cryostat sections examined by the SEM after critical point drying showed severe structural alteration throughout the whole area of cross section (Plate 35) and was probably caused by dehydration immediately after sectioning. Consequently, the

samples prepared in this manner proved unsatisfactory for use in the study of cuticular ultrastructure. Although prior fixation of cuticle samples successfully preserved basic structural integrity, it was found that deformation occurred during sectioning (Plate 36) and these specimens were also unsuitable for further study.

It was possible however, to examine cross sectional formations using whole lice which were simply torn apart after prior osmium tetroxide fixation and critical point drying. The fracture surface produced in this way indicated that the sclerite cuticle failure occurred in a brittle manner (Plates 37 and 38). Pore canals were readily distinguished throughout the sclerite cuticle cross section and structures consistent with those of pore canal filaments were observed within them (Plate 38). In contrast with the results from the TEM studies (see section 3.1.2.1.), the fracture surfaces of sclerite cuticle provided evidence that it did, in fact, possess a layered structure which showed signs of being composed of fibrils (Plate 37).

Membranous cuticle fractured in an entirely different fashion. The outer layers (epicuticle and exocuticle) failed in a brittle manner whilst the underlying endocuticle, composed of macrofibrils, gave some indication of plastic deformation (Plate 39) despite the hardening effects of the osmium fixation. Fracture surfaces through epicuticle and exocuticle layers of membranous cuticle (Plate 39) and all layers of sclerite cuticle (Plate 38) were



Plate 35. Cryostat section of unfixed sclerite which was then critical point dried. Severe structural deformation is evident throughout the area of cross section probably due to uncontrolled dehydration prior to critical point drying.  $\times 10\ 000$ .

Plate 36. Cryostat section of fixed (osmium tetroxide) membranous cuticle which was then critical point dried. Structural deformation has been caused during sectioning by the microtome blade.  $\times 8000$ .

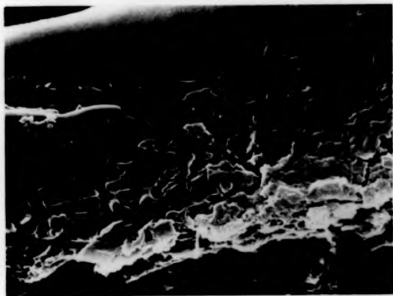
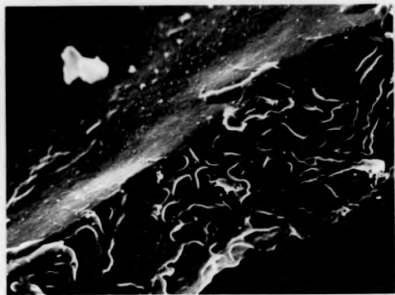


Plate 37. Fracture surface of sclerite cuticle produced by tearing cuticle previously fixed and critical point dried. The endocuticle has a distinct lamellate appearance and there is some indication that it is fibrous (arrow). x 15 000.

Plate 38. Fracture surface of sclerite cuticle prepared as in Plate 37. Pore canals and their filaments are visible and follow relatively direct routes through the endocuticle. x 20 000.

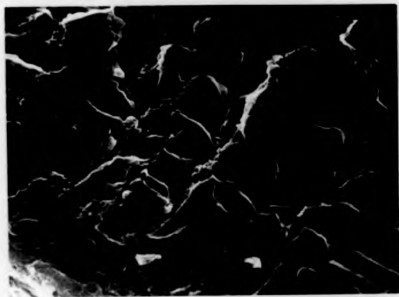
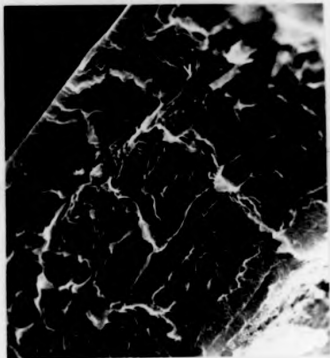
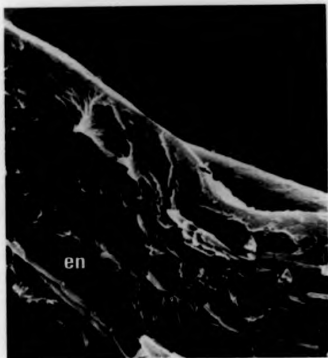


Plate 39. Cross section of membranous cuticle prepared as in Plate 37. The macrofibrillar structure of the endocuticle contrasts with the closely-packed exocuticle and epicuticle. ep, epicuticle; ex, exocuticle; en, endocuticle. x 10 000.

Plate 40. Cross section of membranous cuticle prepared as in Plate 37. The series of holes parallel to the surface and underlying the epicuticle corresponds to the tubular network seen in TEM studies. The striations or channels above the tubular network correspond to epicuticular filaments. x 50 000.



quite similar in appearance and this was consistent with the closely-packed structure of the material involved. The individual macrofibrils comprising the endocuticle of membranous integument were discernible (Plates 39 and 40) and their dimensions ( $400 - 1000 \text{ \AA}$ ) coincided with those estimated from the TEM examination (see section 3.1.2.1.). As observed during the platinum/carbon replication studies (see section 3.1.2.2.), the numbers of macrofibril layers between each lamella was relatively small as a result of successive layers twisting approximately  $45^\circ$  from the next to form a helicoidal arrangement (Plate 39). The tubules located by TEM (see section 3.1.2.1.) at the interface of epicuticle and procuticle were also observed during the SEM examination of sclerite and membranous integument and appeared as a series of holes forming a line parallel to the cuticular surface (Plate 40). Narrow canals were observed traversing the epicuticle perpendicularly to the surface (Plate 40) and corresponded to the epicuticular filaments noted previously (see section 3.1.2.1.).

### 3.1.3.3. Solvent effects on the cuticular wax layer.

Cuticle which was subjected to acetone washing prior to preparation for SEM examination exhibited an extensive loss of cuticular wax mounds from the surface (Plate 41) when compared to the appearance of the untreated cuticular surface (platinum/carbon replication - see section 3.1.2.2.). Despite the removal of many wax mounds, the epicuticular pores remained

Plate 41. The structure of sclerite cuticle which was acetone-washed (10 minutes) prior to fixation ( $\text{OsO}_4$ ) and critical point drying. Epicuticular pores are concealed by wax mounds and a web-like film of wax deposits.  $\times 50\ 000$ .

Plate 42. Surface of sclerite cuticle which was hexane-washed (10 minutes) prior to fixation ( $\text{OsO}_4$ ) and critical point drying. Wax mounds have been removed and epicuticular pores (arrows) are visible through the remaining debris.  $\times 30\ 000$ .



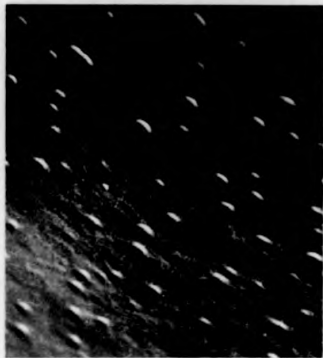
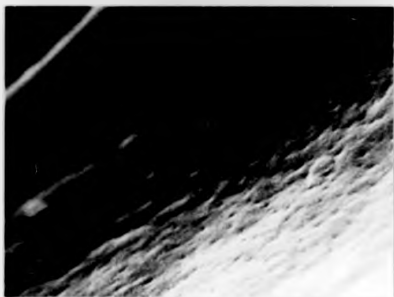


Plate 43. Surface of hexane-washed solerite cuticle as in Plate 42. Epicuticular pores or depressions have been revealed by the complete removal of cuticular wax by the solvent. x 60 000.



concealed by a web-like wax film deposited on the surface (Plate 41).

Hexane acted much more efficiently in dissolving and removing cuticular wax (Plates 42 and 43) so that only insoluble debris remained after washing for 10 minutes (Plate 42). In areas of complete wax removal, surface pores or depressions approximately  $100 \text{ \AA}$  in diameter were clearly seen (Plate 43) which corresponded both in appearance and numerically with the epicuticular structures observed previously in TEM studies (see section 3.1.2.1.).

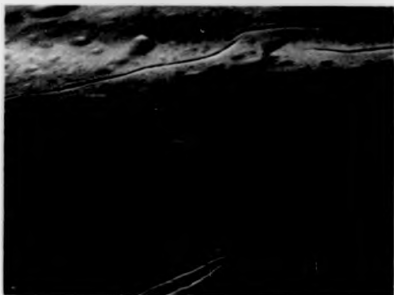
#### 3.1.3.4. Effects of alkali treatment on the surface.

Although some cuticular wax had undoubtedly been removed from the surface, the greater percentage had collected together in large mounds (Plate 44) which were substantially larger than those noted earlier (see section 3.1.2.2.). Removal of wax during alkali treatment was brought about by saponification, but an analysis of the cuticular wax revealed that it was composed of over 75% hydrocarbons which do not respond to such action (see section 3.2.3.2.).

Although the chitinous structure of the cuticle was unaffected by the strong alkali, the epidermal layer was completely removed by digestion and the basal openings of the pore canals were revealed (Plate 45).

Plate 44. Outer surface of sclerite cuticle on which the unsaponified cuticular wax has collected into large mounds after treatment with alkali (KOH). The electron micrograph also shows the surface duct of a dermal gland. x 3500.

Plate 45. Inner endocuticle surface of membranous cuticle after alkali (KOH) treatment during which the epidermal layer was digested. Large basal openings to pore canals are visible throughout the entire surface. x 5000.



- 3.2. Chemical Composition of Leuce Lipids.
- 3.2.1. Comparison of potential extraction solvents.
- 3.2.1.1. Trial thin-layer chromatography (TLC) separations.

Visualisation with iodine vapour of the TLC separations of the solvent outcoular extracts produced positive results for the hexane extract only. These appeared as two faint spots near the solvent front. After sulphuric acid and heat treatment, the two spots became more intense and two more spots (fainter) appeared at distances closer to the origin. Observation of the plate under UV radiation revealed corresponding but much fainter spots produced by the benzene and chloroform extracts while the dimethoxymethane extract produced faint spots at and near the origin which were tentatively identified as phospholipids and cerebrosides.

- 3.2.1.2. Solubility of long chain hydrocarbon ( $n - C_{36}$ ) in potential extraction solvents.

After a period of 10 minutes at room temperature, the hexatriacontane had dissolved in the hexane only. With mild heating of the remaining tubes, the hexatriacontane dissolved in both the benzene and chloroform.

- 3.2. Chemical Composition of Louse Lipids.
- 3.2.1. Comparison of potential extraction solvents.
- 3.2.1.1. Trial thin-layer chromatography (TLC) separations.

Visualisation with iodine vapour of the TLC separations of the solvent cuticular extracts produced positive results for the hexane extract only. These appeared as two faint spots near the solvent front. After sulphuric acid and heat treatment, the two spots became more intense and two more spots (fainter) appeared at distances closer to the origin. Observation of the plate under UV radiation revealed corresponding but much fainter spots produced by the benzene and chloroform extracts while the dimethoxymethane extract produced faint spots at and near the origin which were tentatively identified as phospholipids and cerebrosides.

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3.2.2. Extraction of louse lipids.

3.2.2.1. Cuticular lipid yields from extractions of whole lice.

Table 5 shows a comparison of both fresh and dry body weights of male and female adult lice. According to the results, the adult female was found to be significantly heavier than the corresponding male (46.96% heavier) and this relationship was maintained when dry weights were compared (48.58% heavier).

The weight of the female adult louse decreased by an average of 66.12% upon drying whilst that of the male adult decreased by an average of 66.48%, illustrating the high water content of the adult lice.

A quantitative comparison of the wax yields from hexane extractions is shown in Table 6 . On average, the female adult yielded approximately 20% more wax through hexane extraction than the male adult. However, when compared as percentages of body weights (both fresh weights and dry weights), the average yield of cuticular wax from the male adult exceeded that of the female adult by approximately 20%.

Table 5

Comparison of fresh and dried adult weights

Female lice (adult, 2 - 3 days)			Male lice (adult, 2 - 3 days)		
numbers of lice	fresh weight mg/louse	dried weight mg/louse	numbers of lice	fresh weight mg/louse	dried weight mg/louse
200	1.779	0.569	200	1.125	0.365
200	1.659	0.550	200	1.157	0.322
540	1.299	0.445	759	0.908	0.307
435	1.411	0.519	416	0.993	0.406
Average weight mg/louse	1.537	0.521	Average weight mg/louse	1.046	0.350

Table 6

## Comparison of cuticular wax yields

numbers and sex of lice	mass of extracted wax mg	mass wax per louse $\mu$ g/louse	wax as % of fresh weight	wax as % of dry weight
435 ♀	0.10	0.23	0.0163	0.0488
540 ♀	0.11	0.20	0.0157	0.0458
416 ♂	0.08	0.19	0.0193	0.0580
759 ♂	0.14	0.17	0.0188	0.0556

### 3.2.2.2. Examination of cuticular lipid extraction technique (from whole lice).

Sampling of blood from the site of lice feeding on the rabbit indicated a maximum level of 275 counts/40  $\mu$ l blood specimen (Table 7 ). The ratio of scintillations from the extracted cuticular wax and labelled oleic acid to the scintillations from the total labelled oleic acid consumed by the lice represented the fraction of internal lipids removed simultaneously with the cuticular lipids during a 10 minute extraction period.

$$\begin{aligned}
 \text{i.e. } & \frac{\text{internal lipids removed during cuticular lipid extraction}}{\text{total internal lipids}} \\
 &= \frac{\text{cpm (cuticular lipids + internal labelled oleic acid)}}{\text{cpm (remaining internal labelled oleic acid + labelled oleic acid extracted with cuticular lipids)}} \\
 &= \frac{1272 - \text{background}}{46270 - \text{background} + 1272 - \text{background}} \\
 &= \frac{1247}{47492} \\
 &= 0.0262 \\
 &= 2.62\%
 \end{aligned}$$

The 10 minute extraction of the lice colony (approximately 10,000 lice) produced 1.5 mg of cuticular wax which was equivalent to 0.15  $\mu$ g per insect (see section 3.2.2.1.).

Table 7

Scintillation counts on labelled/unlabelled samples

Sample	Scintillations cpm <sup>a</sup>
blood <sup>b</sup> (unlabelled)	21
blood <sup>b</sup> (after louse feeding, 20 minutes)	275
cuticular lipids + internal labelled oleic acid	1272
remaining internal labelled oleic acid	46270
background	25

a. average scintillation counts per minute from 5 observations.

b. blood serum 40  $\mu$ l.

- 3.2.3. Thin-layer chromatographic (TLC) analysis of lipid extracts.
- 3.2.3.1. Qualitative TLC analysis and comparison of cuticular lipids from whole lice and cast skins.

Detection with iodine vapour of the cuticular lipids from whole lice revealed the presence of six lipid classes (Skipski et al., 1965) and the speed and intensity with which the hydrocarbon spot appeared, indicated some unsaturation. Sulphuric acid and heat treatment did not reveal further spots (Plate 46). During heating, two of the spots first appeared red before turning black which was characteristic of the behaviour of cholesterol and cholesteryl esters (Mangold, 1965). The six lipid classes were tentatively identified by comparison with authentic standards chromatographed simultaneously on the plate and are listed below in decreasing order of intensity.

- i hydrocarbons
- ii cholesteryl esters
- iii triglycerides
- iv 1, 3 - diglycerides
- v cholesterol
- vi fatty acids.

Eight lipid classes were detected with iodine vapour in the cuticular lipid extract from cast skins and further treatment with sulphuric acid and heat did not reveal any additional classes. The spots appeared in the following decreasing order of intensity.

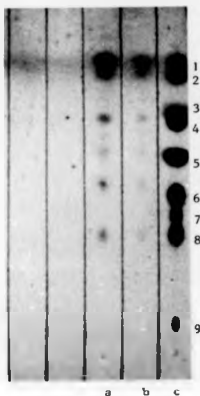


Plate 46. TLC separation of cuticular louse lipids.

- a,b - louse lipids  
 c - reference standards
1. hexatriacontane
  2. cholesteryl stearate
  3. methyl stearate
  4. triolein
  5. oleic acid
  6. 1,3-diolein
  7. 1,2-diolein
  8. cholesterol
  9. 1- and 2-monoolein

- i phospholipids/cerebrosides
- ii fatty acids
- iii cholesteryl esters
- iv cholesterol
- v hydrocarbons
- vi 1 - and 2 - monoglycerides
- vii triglycerides
- viii 1, 3 - diglycerides.

3.2.3.2. Quantitative TLC analysis (by weight) of cuticular lipids.

Hydrocarbons were found to constitute almost three-quarters by weight of the louse cuticular lipids (Table 8 ). Cholesteryl esters formed the second most abundant lipid class but at approximately one-tenth the weight of the hydrocarbons. The occurrence of the remaining lipids i.e. triglycerides, 1, 3 - diglycerides, cholesterol and fatty acids was substantially lower, between 5.79% (triglycerides) and 3.87% (fatty acids).



Table 8

Composition of cuticular louse lipids (by weight)

Lipid Class	Mass mg		Percentage of Total Lipids		Average Percentage
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
hydrocarbons	2.700	4.210	72.835	74.712	73.77
cholesteryl esters	0.259	0.463	6.987	8.216	7.60
triglycerides	0.237	0.292	6.393	5.182	5.79
fatty acids	0.162	0.198	4.370	3.372	3.87
1, 3-diglycerides	0.201	0.223	5.422	3.957	4.69
cholesterol	0.148	0.249	3.992	4.419	4.21
Total	3.707	5.635	99.999	99.858	99.93

3.2.3.3. Quantitative TLC analysis (by densitometer) of cuticular lipids/internal lipids/faecal lipids.

The TLC separations of the various louse lipids (Plate 47 ) were scanned with a densitometer and the percent composition estimated by comparing weights of the chromatogram peaks cut from the recorder trace (Table 9 ). The concentration of hydrocarbons was highest in the cuticular lipids (55.2%) and relatively high in the whole body (17.1%). Cholesteryl esters constituted the most abundant lipid class in faecal lipids (59.2%) while the levels in cuticular lipids (13.7%) and whole body (17.7%) were quite similar.

The concentrations of triglycerides and fatty acids in all three samples were relatively low except for the fatty acids of the whole body lipids (40. 2%) which comprised the predominant lipid class of that mixture.

Levels of 1, 3 - diglycerides and monoglycerides throughout were low while cholesterol levels were comparatively high, i.e. cuticular wax (13.2%), faeces (19.1%) and whole body (13.2%).

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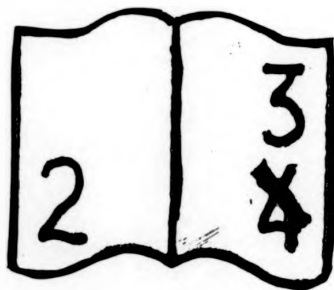




Plate 47. TLC separation of louse lipids.

- a,e - ♂ internal lipids
- b - ♂ + ♀ faecal lipids
- c - ♂ + ♀ cuticular lipids
- d - ♀ internal lipids
- f - ♂ + ♀ faecal lipids

Table 9

Composition of louse lipids (using TLC - densitometer)

Lipid source	Lipid class	% composition of total
extracted outicular wax	hydrocarbons	55.2
	cholesteryl esters	13.7
	triglycerides	11.6
	fatty acids	1.9
	1,3-diglycerides	4.4
	cholesterol	13.2
faeces	hydrocarbons	6.4
	cholesteryl esters	59.2
	triglycerides	3.7
	fatty acids	9.9
	cholesterol	19.1
	monoglycerides	1.7
whole body	hydrocarbons	17.1
	cholesteryl esters	17.7
	triglycerides	7.2
	fatty acids	40.2
	1,3-diglycerides	1.5
	cholesterol	13.2
	monoglycerides	3.1

### 3.2.3.4. TLC analysis of digestion products of labelled fatty acid (oleic acid).

The hydrocarbons (73.05%) possessed the highest level of radioactivity of the lipid classes of the cuticular wax (Table 10) and also that of the female whole body (36.59%) but the level was much lower in the faecal matter (6.28%). The value for the cholesteryl esters (73.82%) in the faeces was similar to that of the hydrocarbons in the cuticular wax, while it was also relatively high in the whole body (19.88%) but low in the cuticular wax (5.42%). The radioactive levels for other lipid classes (i.e. triglycerides, fatty acids, diglycerides, cholesterol and monoglycerides) were not significantly high (Table 10) with the exception of fatty acids in the whole body (29.65%).

Using the scintillation data it was possible to calculate the mean volume of blood taken per insect during the 10 minute feeding period.

Assuming virtually all the radioactivity was recovered from the 20 fed adult lice, then

scintillation (cp10m) from cuticular lipids =	162.4
scintillation (cp10m) from faecal lipids =	245.2
scintillation (cp10m) from whole body lipids =	458.6
Total scintillation (cp10m)	= 866.2

scintillation (cpm) from blood (40  $\mu$ l) during feeding = 238(mean)

scintillation (cp/0m) from blood (1  $\mu$ l) during feeding =  $\frac{2380}{40}$

Total blood volume ( $\mu$ l) taken by 20 insects =  $\frac{866.2 \times 40}{2380}$

Average blood volume per insect =  $\frac{866.2 \times 40}{20 \times 2380} = 0.7278 \mu$ l

This result was compared with that obtained from weighing a known number of lice (120) before and after feeding for 10 minutes. The density of the blood ( $1.0698 \text{ g ml}^{-1}$ ) was calculated by weighing a known volume of blood (50  $\mu$ l) from the rabbit on which the lice were fed.

mass of container with 120 unfed lice = 13.2448 g

mass of container with 120 fed lice = 13.3416 g

mass of blood ingested by 120 lice = 0.0968 g

mass of blood ingested by 1 louse(mean) = 0.000806g

density =  $\frac{\text{mass}}{\text{volume}}$

volume (mean) of blood per louse =  $\frac{0.0968 \times 1}{120 \times 1.0698} \text{ ml}$

= 0.000754 ml

= 0.754  $\mu$ l

From the scintillation data it was also possible to estimate more accurately the numbers of lice used in the original experiment to examine the extraction technique (see section 2.2.6.1.).

Table 10

Metabolic products of tritiated oleic acid

Lipid source	Lipid class	Scintillation cp 10m <sup>a</sup>	% composition of total
extracted cuticular wax	hydrocarbons	193.6	73.03
	cholesteryl esters	83.8	5.42
	triglycerides	92.6	10.83
	fatty acids	86.6	7.14
	cholesterol	80.8	3.57
faeces	hydrocarbons	90.4	6.28
	cholesteryl esters	256.0	73.82
	triglycerides	86.6	4.73
	fatty acids	97.8	9.30
	cholesterol	89.4	5.87
whole body (female)	hydrocarbons	242.8	36.59
	cholesteryl esters	166.2	19.88
	triglycerides	91.2	3.53
	fatty acids	211.0	29.65
	1,3 diglycerides	89.0	3.05
	cholesterol	94.0	4.14
	monoglycerides	89.4	3.14

a average scintillation counts per 10 minutes from 5 observations

b calculated after subtraction of background (75 cp10m)



From above:

Total scintillation (cp10m) from 20 lice = 866.2

From 2.2.6.1.

Total scintillation (cpm) from x lice = 47492

Total scintillation (cp10m) from x lice = 474920

$$\begin{aligned}
 x \text{ (numbers of lice used)} &= \frac{20 \times 47492}{1 \times 866.2} \\
 &= 10,965 \text{ lice}
 \end{aligned}$$

### 3.2.3.5. Degree of saturation of cuticular hydrocarbons.

The hydrocarbons separated into two distinct spots corresponding with the saturated and mono-<sup>unsaturated</sup> reference spots, hexatriacontane and 1 - octadecene respectively. From these results, both saturated and unsaturated hydrocarbons appeared to be present in the cuticular lipids and the unsaturated hydrocarbons were mono-unsaturated.

3.2.4. Gas-liquid chromatographic (GLC) analysis of lipid extracts.

3.2.4.1. GLC analysis of cuticular hydrocarbons.

The chromatograms produced by the cuticular hydrocarbons from adult lice separated according to sex were identical (GLC peak numbers 1 to 36) and contained four major peaks which gave the appearance of forming a series (Fig. 6 ). Proportions and retention times of the corresponding minor GLC peaks from both samples coincided exactly. Separation of saturated from unsaturated hydrocarbons (see section 2.2.4.3.) indicated that the cuticular louse mixture contained eight mono-unsaturated hydrocarbons (peak nos. 4, 9, 15, 18, 21, 24, 27 and 33 all absent in Fig. 7 ). Removal of straight chain hydrocarbons from the total hydrocarbons with molecular sieve 5A (Fig. 8 ) enabled the structural character of the hydrocarbons to be determined i.e. whether branched or unbranched, while co-chromatography with n - alkane standards confirmed many of the identifications. A linear plot of retention time versus carbon numbers revealed the presence of three series of hydrocarbons; A - normal alkanes, B - normal alkenes and C - tentatively identified as mono methylalkanes (Fig. 9 ).

As the subsequent tests proved, one major component (peak no. 27) was mono-unsaturated (series B) and did not form a

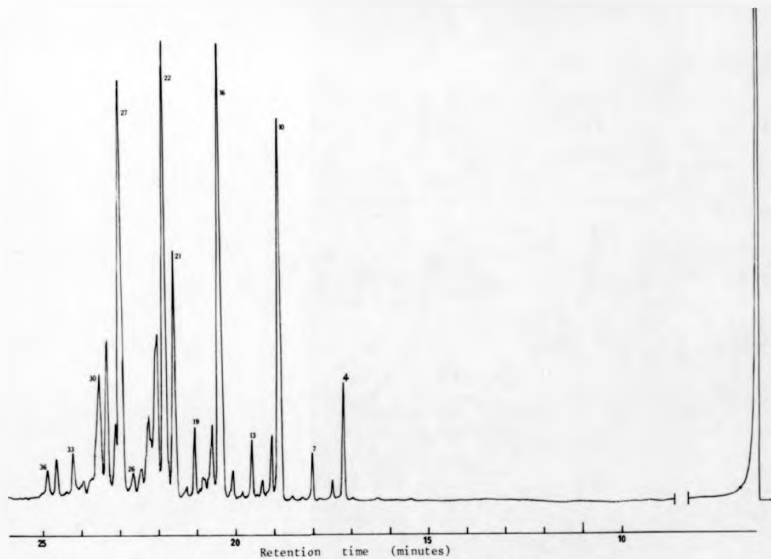


Figure 6. Gas chromatogram of the cuticular hydrocarbons of the human body louse.

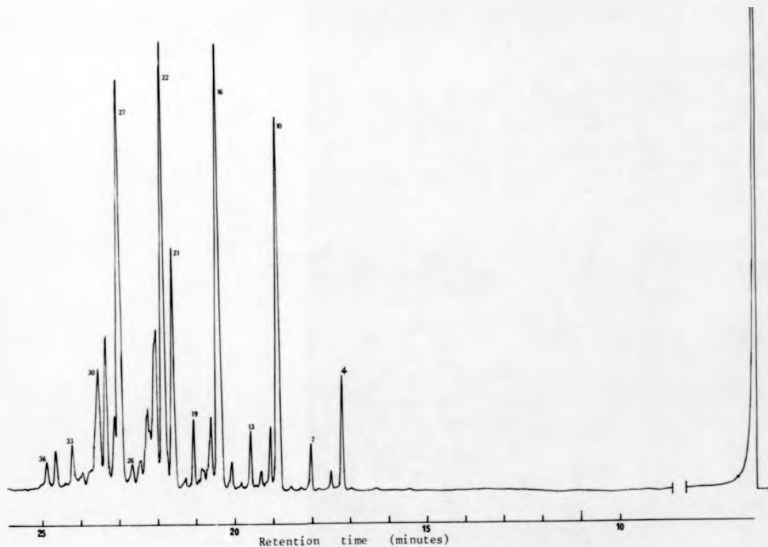


Figure 6. Gas chromatogram of the cuticular hydrocarbons of the human body louse.

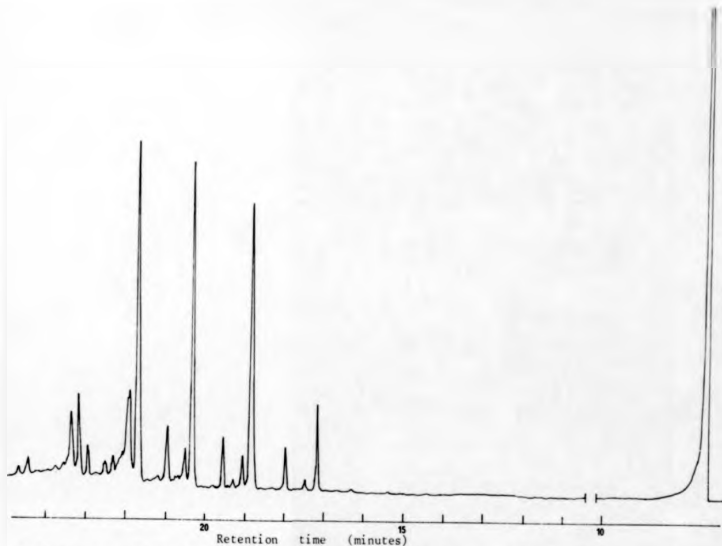


Figure 7. Gas chromatogram of cuticular hydrocarbons of the louse after removal of unsaturated components.

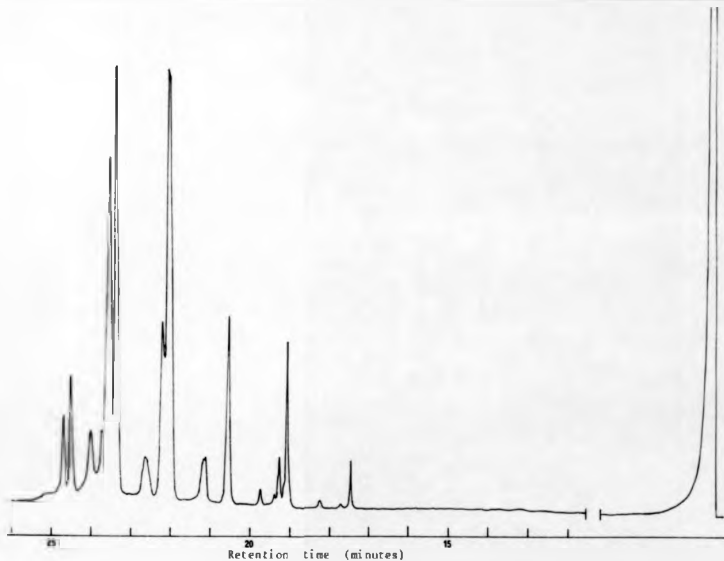


Figure 8. Gas chromatogram of cuticular hydrocarbons of the louse after removal of straight chain components.

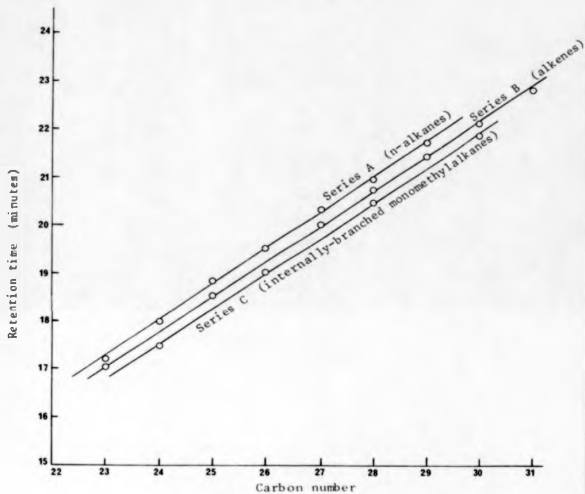


Figure 9. Plot of retention time vs carbon number of the cuticular hydrocarbons of the louse.

series with the three other major peaks (nos. 10, 16, 22) which were identified as n - alkanes (series A). GLC analysis following the periodate-permanganate oxidation (see section 2.2.4.6.) of the unsaturated hydrocarbons produced two main fatty acid methyl ester peaks which contained 15 and 16 carbons in the fatty acid portion respectively. This result agreed with the reaction products expected of peak 27 which appeared to contain 31 carbons in a straight chain. Unfortunately, no other peaks of significance appeared so it was impossible to deduce the structure of the second most abundant alkene (peak no. 21).

Table 11 (page 186 ) shows the completed analysis of the hydrocarbons in which the methylalkane structures were determined from their mass spectra as well as retention times.

#### 3.2.4.2. Gas chromatography/Mass spectroscopy (GC/MS) analysis of cuticular hydrocarbons.

In each case, the identity of the n - alkanes, as determined by GLC techniques (see section 2.2.4.2.) was confirmed by the results of the mass spectral analysis. The mass spectra obtained with Electron Ionisation (E.I.) was more readily interpreted than that obtained with Chemical Ionisation (C.I.). This was due, in part, to the lower sensitivity at which the system was operated for the C.I. mode.



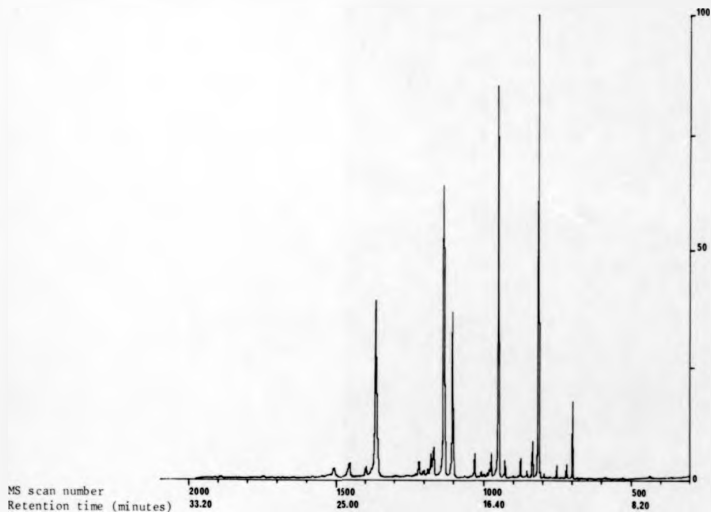


Figure 10. GC/MS (E.I.) total ion trace of cuticular hydrocarbons of the louse.

Peak No.	E.C.L.	Series	Mean % Composition		Hydrocarbons
			Hydrocarbons	Wax	
1	21.0	A	T <sup>b</sup>	T	n - heptacosane
2	22.0	A	T	T	n - docosane
3	22.7	B	T	T	unsaturated n-tricosane
4	23.0	A	2.0	1.8	n - tricosane
5	23.3	C	0.3	0.2	11 - methyltricosane
6	23.7	-	T	T	Unknown
7	24.0	A	0.9	0.6	n - tetracosane
8	24.3	-	T	T	Unknown
9	24.6	B	0.2	0.1	Unsaturated n-pentacosane
10	25.0	A	14.0	10.3	n - pentacosane
11	25.3	C	1.2	0.9	11 - methylpentacosane + 13 - methylpentacosane
12	25.6	D	0.3	0.2	11, 15-dimethylpentacosane
13	26.0	A	1.0	0.7	n - hexacosane
14	26.3	-	T	T	Unknown
15	26.6	B	0.8	0.6	Unsaturated n - heptacosane
16	27.0	A	15.1	11.1	n - heptacosane
17	27.3	C	1.7	1.2	11-methylheptacosane + 13-methylheptacosane
18	27.6	B	0.7	0.5	Unsaturated n-octacosane
19	28.0	A	1.7	1.2	n - octacosane
20	28.3	-	0.5	0.4	Unknown
21	28.7	B	7.8	5.7	Unsaturated n-nonacosane
22	29.0	A	15.6	11.5	n - nonacosane

Peak No.	E.C.L.	Series	Mean % Composition		Hydrocarbons
			Hydrocarbons	Wax	
23	29.3	C	4.9	3.6	7 - methylnonacosane + 9 - methylnonacosane + 11 - methylnonacosane
24	29.7	B	2.4	1.8	Unsaturated n-triacontane + unknown
25	30.0	A	0.5	0.4	n - triacontane
26	30.3	-	0.4	0.3	Unknown
27	30.8	B	15.4	11.3	15 - hentriacontene
28	31.0	A	1.0	0.8	n - hentriacontane
29	31.3	C	3.3	2.4	9 - methylhentriacontane + 11-methylhentriacontane + 13-methylhentriacontane
30	31.6	D	3.7	2.7	9,19-dimethylhentriacontane + 11,21-dimethylhentriacontane
31	32.0	A	T	T	n - dotriacontane
32	32.3	-	0.5	0.4	Unknown
33	32.7	B	0.8	0.6	Unsaturated n - tritriacontane
34	33.0	A	T	T	n - tritriacontane
35	33.3	C	0.7	0.5	13 - methyltritriacontane
36	33.6	D	0.7	0.5	Possible dimethylalkane mixture

a-E.C.L. Equivalent Chain Length.

b-T < 0.1% of total hydrocarbons.

Table 11. Composition of cuticular hydrocarbons

Hence, those hydrocarbons which were present at lower levels produced seriously depleted spectra. Several of the  $n$  - alkanes, however, were quite abundant and their spectra were significantly enhanced by the use of C.I. (Fig. 11 a/b). The data system was particularly useful in allowing a search to be conducted through the MS scans whereby  $n$  - alkanes ( $C_n H_{2n+2}$ ) and mono-unsaturated  $n$  - alkenes ( $C_n H_{2n}$ ) were detected on the basis of their molecular ions (Fig. 12). Table 11 shows the completed analysis of the cuticular hydrocarbons  $n$  - alkanes (51.6%) comprised over half of the cuticular hydrocarbons and formed an homologous series (Series A, see 3.2.4.1.) with carbon numbers ranging from 21 to 33. Odd-numbered components (47.7%) predominated and, together,  $n$  - pentacosane (14.0%),  $n$  - heptacosane (15.1%) and  $n$  - nonacosane (15.6%) accounted for almost half of the cuticular hydrocarbons (44.7%). The mass spectrum (Fig. 13) of  $n$  - nonacosane (Peak no. 22) illustrates the fragmentation pattern typical of long chain  $n$  - alkanes. The largest peak in each cluster is at  $m/e$  ( $C_n H_{2n+1}$ ), indicating all fragmentations to be straight chain primary ions. The molecular ion ( $C_n H_{2n+2}$ ) was present ( $m/e$  408) but weak while the most intense fragment ions occurred at  $C_3$ ,  $C_4$  and  $C_5$  with intensities decreasing along a smooth curve formation down to  $M - C_2H_5$ . Fragment ion,  $M - CH_3$  was characteristically missing from the spectrum.

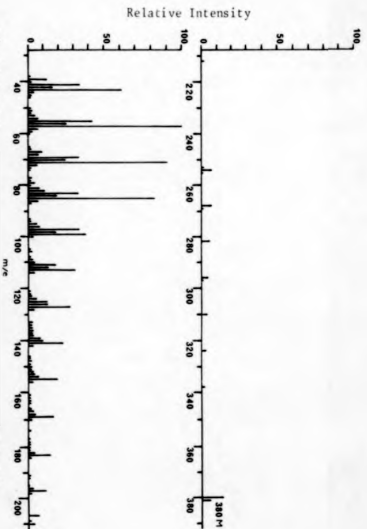
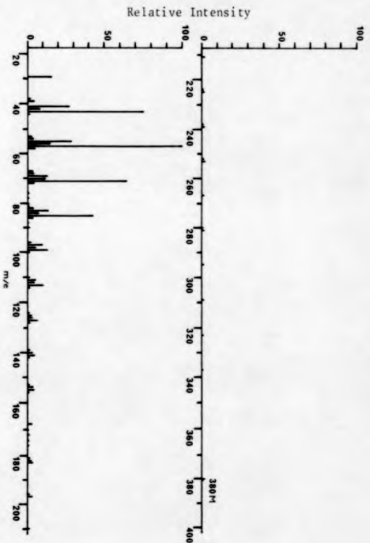


Figure 11 a,b. Comparison of E.I. and C.I. mass spectra of n-heptacosane.

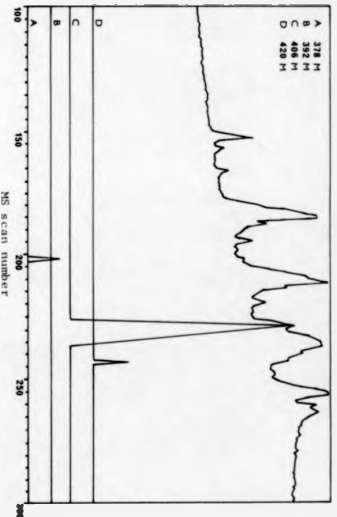


Figure 12. NS data search for unsaturated alkenes  $n\text{-C}_{27}$ ,  $n\text{-C}_{28}$  and  $n\text{-C}_{30}$ .

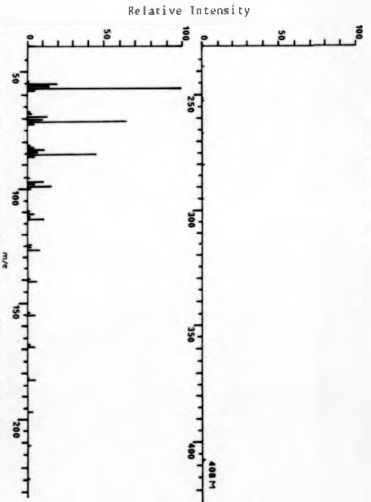


Figure 13. Mass spectrum of GLC peak 22,  $n\text{-nonacosane}$ .

Eight n - alkenes (Series B, 26.4%) comprised over a quarter of the cuticular hydrocarbons with 15 - hentriacontene (15.4%) the most abundant of the series. Upon GLC separation of the hydrocarbons, each n - alkene was eluted immediately prior to the n - alkane of the same carbon number and the mass spectrum of each n - alkene contained a molecular ion at  $m/e$  ( $C_n H_{2n}$ ). Fragmentations patterns throughout all n - alkene mass spectra were identical and the mass spectrum (Fig. 14) of 15 - hentriacontene (Peak no. 27) displays the fragmentation typical of this series. The base peak was at  $m/e$  57 followed by two peaks of equal intensity at  $m/e$  83 and  $m/e$  97; then another, of lesser intensity, at  $m/e$  69. After  $m/e$  97, the fragment ion intensities decreased along a smooth curve to  $M - C_2H_4$ . As the n - alkene chains were long, it was impossible to locate the position of the double bond from the MS data due to the facile migration in the fragments of the double bond. However, the position had already been established for 15 - hentriacontene using GLC techniques (see section 3.2.4.1.) but not for other n - alkenes.

Series C compounds (12.1% of cuticular hydrocarbons) were not removed by treatment with molecular sieve 5A (see Materials and Methods section 2.2.4.2.3.) thus revealing the branched configuration of their structure. For each compound, a difference of 0.7 was observed between the equivalent chain length (E.C.L.) and the carbon number, indicating that the

compounds might be monomethylalkanes, each containing the methyl branch in a central position in the molecule (Nelson et al, 197 ). Further evidence was found from examination of the mass spectral data in which molecular ions (M) were missing but peaks invariably found at M-15 and M-27 (Fig. 15 Peak no. 17). Where fragmentation ion doublets occurred, the even mass peaks ( $C_n H_{2n}$ ) equalled or exceeded the corresponding odd mass peaks ( $C_n H_{2n+1}$ ) in intensity. This inferred that the alkane contained a single methyl branch attached at a position which allowed the formation, upon fragmentation, of secondary ions on which the straight chain tails comprised at least 7 carbon atoms (McCarthy et al, 1968). The mass spectrum of GLC Peak no. 17 (Fig. 15 ) contained ion doublets at m/e 168/169 ( $C_{12}$ ), m/e 196/197 ( $C_{14}$ ), m/e 224/225 ( $C_{16}$ ) and m/e 252/253 ( $C_{18}$ ) with predominant even mass peaks and low intensity peaks at m/e 365 (M - 29) and m/e 379 (M - 15). This fragmentation pattern indicated that the GLC peak consisted of two monomethylalkanes of the same carbon number (28), and both with a central methyl branch (i.e. at carbons 11 and 13 for the respective methylalkanes). The compounds were identified as 11 - and 13 - methylheptacosane. Other members of the series were examined similarly and identifications made (see Table 11).

Evidence was found to suggest the existence of a fourth series (series D) of hydrocarbons consisting of dimethylalkanes.



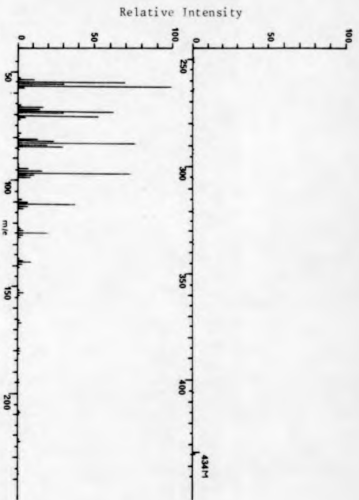


Figure 14. Mass spectrum of GLC peak 27, 15-hentriacontene.

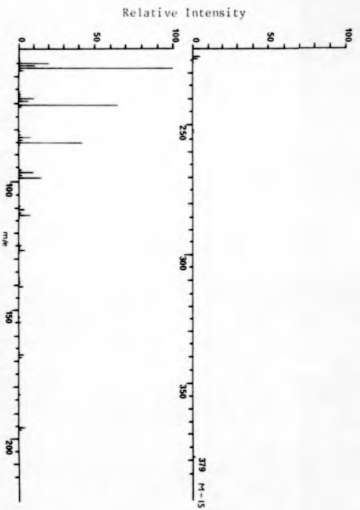


Figure 15. Mass spectrum of GLC peak 17, 11 + 13-methylheptacosane.

GLC peaks 12, 30 and 36 had ECL values of 25.6, 31.6 and 33.6 respectively which, together with their mass spectra and non-removal with molecular sieve 5A, indicated that they were di- or tri-methylalkanes. The mass spectrum of GLC peak 12 (Fig. 16) contained neither the molecular ion peak nor the more usual peaks at  $(M - 15)$  and  $(M - 29)$  due to the low concentration of the compound. Fragmentation ion doublets occurred  $m/e$  168/169 ( $C_{12}$ ),  $m/e$  182/183 ( $C_{13}$ ),  $m/e$  210/211 ( $C_{15}$ ) and  $m/e$  238/239 ( $C_{17}$ ) with the  $C_{12}$  and  $C_{17}$  ion doublets of almost equal intensity. The  $C_{12}$  ion was considered to be a secondary fragment ion since its even mass peak ( $m/e$  168) intensity exceeded that of its odd mass peak ( $m/e$  169), while the  $C_{17}$  ion was the corresponding primary fragment ion with its odd mass peak ( $m/e$  239) intensity predominating due to the methyl branch at carbon 15 (Fig. 17).

The significance of ion doublets  $m/e$  182/183 ( $C_{13}$ ) and  $m/e$  210/211 ( $C_{15}$ ) in this depleted mass spectrum was difficult to establish. Their presence was possibly due to fragmentation within the central region of the molecule as shown in Fig. 17 but it would have been expected that  $m/e$  211 (odd mass peak) exceed  $m/e$  210 (even mass peak) in that case. Since, in fact, the opposite occurred, the ion attributed to  $m/e$  210/211 might have been a secondary fragment ion produced in conjunction with ion doublet  $m/e$  182/183 from the fragmentation of the mono-methylalkane (12 methylpentacosane) shown in Fig. 18.

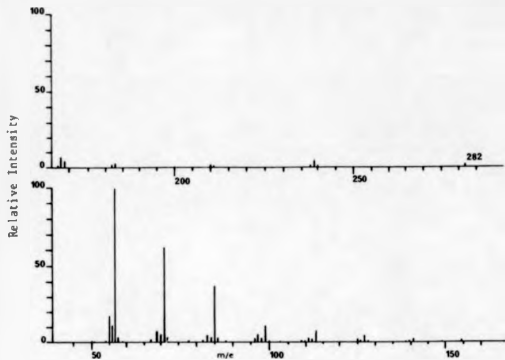


Figure 16. Mass spectrum of GLC peak 12, 11,15-dimethylpentacosane.

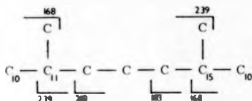


Figure 17. Structure for GLC peak 12.

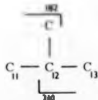


Figure 18. Alternative structure for GLC peak 12.

The structure of this compound satisfied the mass spectral information; however, the GLC peak was eluted shortly after GLC peak 11 which comprised the unresolved monomethylalkanes 11- and 13-methylpentacosane. If 12-methylpentacosane was present, it was unlikely to be eluted separately. GLC peak 12 was therefore tentatively identified as 11, 15-dimethylpentacosane.

From the chromatogram of the hydrocarbons, the appearance of the second peak (GLC peak 30) of the Series (D) suggested that the peak contained more than one compound. The complexity of its mass spectrum (Fig. 19) certainly supported this view, and the spectrum was interpreted in the same manner as that of the previous dimethylalkane.

Tentative identifications (Fig. 20) were made which suggested that at least two dimethylalkanes (9, 11- and 11, 21-dimethylhentriacontane) were present; however, neither of these compounds could account for the branched primary fragment ion at  $m/e$  210/211.

Fig. 21 shows the incomplete mass spectrum produced from another position in GLC peak no. 30. The ion doublet at  $m/e$  210/211 may be the result of a primary fragment ion of the same fragmentation which produced a secondary fragment ion at  $m/e$  280/281 (Fig. 22).

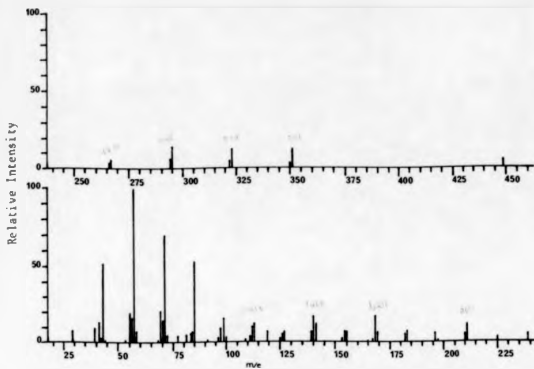
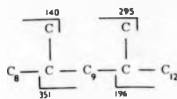
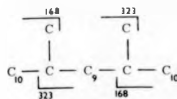


Figure 19. Mass spectrum of GLC peak 30 (First position on GLC peak).



9,19-dimethylhentriacontane



11,21-dimethylhentriacontane

Figure 20. Tentative structures for GLC peak 30.

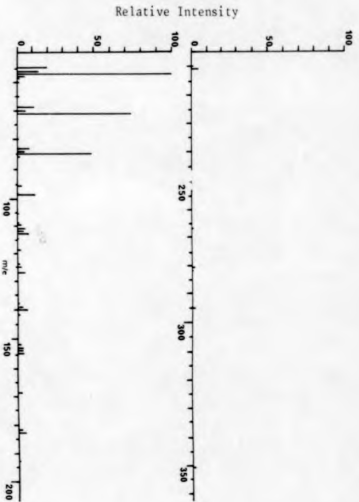
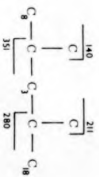


Figure 21. Mass spectrum for GLC peak 30 (Second position on GLC peak).



9, 13-dimethylhentriacontane

Figure 22. Tentative structure for GLC peak 30.

The third GLC peak in Series D (GLC peak 36) produced a grossly depleted mass spectrum due to low concentration and extended retention time and further identification was prevented.

3.2.4.3. GLC analysis of internal hydrocarbons/faecal hydrocarbons.

With the exception of the 15-hentriacontene levels, cuticular and faecal hydrocarbons were closely comparable; however, differences were noted when both were compared with internal hydrocarbons (Table 12 ). Differences were also observed within the internal hydrocarbon components from male and female adult lice. While n-pentacosane was the most abundant hydrocarbons in both adult sexes (Table 12 ), the level in the adult female (25.8%) far exceeded that in the adult male (15.8%). Conversely, the levels in the adult males of n- heptacosane (10.4%), n - octacosane (9.8%) and 15-hentriacontene (11.0%) were much higher than those in the adult females, (1.6% and 0.1% and 1.5% respectively).

Comparison of internal and cuticular hydrocarbons revealed that n - heneicosane and n - docosane, present at trace levels in the cuticular wax, were appreciably more abundant in the internal tissues of adult females (2.3% and 2.0% respectively) and males (0.6% and 1.1% respectively). Major cuticular

Peak No.	Hydrocarbon Component(s)	Percentage Composition of Hydrocarbons				
		Cuticular <sup>a</sup>	Cuticular <sup>b</sup>	Internal <sup>b</sup>	Internal <sup>b</sup>	Fascial <sup>b</sup>
		♂ + ♀	♂ + ♀	♂	♀	♂ + ♀
1	n - heneicosane	T <sup>a</sup>	T	0.6	2.3	T
2	n - docosane	T	T	1.1	2.0	T
3	n - tricosane	T	T	T	T	T
4	n - tricosane	2.0	2.6	10.1	12.3	2.9
5	11-methyltricosane	0.3	0.6	1.4	2.0	0.6
6	Unknown	T	T	T	T	T
7	n - tetracosane	0.9	1.0	2.7	4.6	0.9
8	Unknown	T	T	0.2	0.2	T
9	n - pentacosane	0.2	0.2	0.5	0.4	0.2
10	n - pentacosane	14.0	14.5	15.8	25.8	17.5
11	11 + 13-methylpentacosane	1.2	1.7	3.1	5.4	1.8
12	11, 15-dimethylpentacosane	0.3	0.5	0.6	0.8	0.3
13	n - hexacosane	1.0	1.5	3.7	1.7	1.5
14	Unknown	T	T	0.3	0.5	0.5
15	n - heptacosane	0.8	1.0	2.0	2.5	0.7
16	n - heptacosane	15.1	15.6	10.4	1.6	19.3
17	11 + 13-methylheptacosane	1.7	1.7	3.0	7.5	2.0
18	n - octacosane	0.7	0.6	0.6	0.7	0.3
19	n - octacosane	1.7	2.1	9.8	T	3.1
20	Unknown	0.5	0.3	0.3	1.0	0.2



Peak No.	Hydrocarbon Component(s)	Percentage Composition of Hydrocarbons				
		Cuticular <sup>a</sup> ♂ + ♀	Cuticular <sup>b</sup> ♂ + ♀	Internal <sup>b</sup> ♂	Internal <sup>b</sup> ♀	Faecal <sup>b</sup> ♂ + ♀
21	n - nonacosane	7.8	8.0	15.1	10.2	7.3
22	n - nonacosane	15.6	15.8	T	T	20.7
23	7 + 9 + 11-methylnonacosane	4.9	3.4	3.8	9.4	4.7
24	n - triacontene + unknown	2.4	1.4	1.1	2.0	1.8
25	n - triacontane	0.5	0.5	T	T	0.4
26	Unknown	0.4	0.3	T	T	0.3
27	15 - hentriacontene	15.4	15.3	11.0	1.5	7.0
28	n - hentriacontane	1.0	1.5	T	T	0.3
29	9 + 11 + 13-methyl-hentriacontane	3.3	3.5	0.6	0.3	2.7
30	9, 19 + 11, 21 + 9,13-dimethylhentriacontane	3.7	2.9	1.2	3.9	1.8
31	n - dotriacontane	T	T	T	T	T
32	Unknown	0.5	0.2	T	T	T
33	n - tritriacontene	0.8	1.0	T	T	T
34	n - tritriacontane	T	T	T	T	T
35	13-methyltritriacontane	0.7	1.0	T	T	0.3
36	Possible dimethyl-alkanes	0.7	0.8	0.2	0.3	0.2

a - Estimations from areas given by electronic integration

b - Estimations using peak heights

c - T  $\leq$  0.1 % of hydrocarbons.

Table 12. Comparison of cuticular/internal/faecal hydrocarbons

hydrocarbons, n - heptacosane (15.6%), n - nonacosane (15.8%) and 15 - hentriacontene (15.7%) were present at much lower levels in adult females (1.6%, 0.1% and 1.5% respectively) and this trend was repeated for n - nonacosane in the adult males (0.1%). In contrast with its low occurrence in cuticular wax, n - octacosane (2.1%) was found to be more abundant in adult males (9.8%).

#### 3.2.4.4. GLC analysis of cuticular cholesteryl esters.

Table 15 lists the main fatty acids derived from the cholesteryl esters of the cuticular wax after identifications had been carried out by comparison of retention times of standards and unknowns and by co-chromatography. Although the degree of saturation of the fatty acids was not established, the fractional values for the equivalent chain lengths can probably be attributed to unsaturated carbon-carbon bonds within the chains.

The fatty acid chains ranged from 14 carbons (myristic acid, 1.8%) to 25 carbons (pentacosanoic acid 30.7%) with the latter being most predominant. Palmitic acid (16.0%) and oleic acid (9.6%) followed respectively in order of abundance; however, with cholesteryl esters accounting for less than 8% of the total wax, cholesteryl pentacosanoate was the only cholesteryl ester present at a significant level (2.3% of wax).

Table 13

Fatty acid composition of cuticular  
cholesteryl esters

Fatty acid B.C.L. <sup>a</sup>	Mean % Composition	
	of cholesteryl esters <sup>b</sup>	of wax
14.0(C <sub>14:0</sub> )	1.8	0.1
16.0(C <sub>16:0</sub> )	16.0	1.2
17.6(C <sub>18:2</sub> )	1.5	0.1
17.7(C <sub>18:1</sub> )	9.6	0.7
18.0(C <sub>18:0</sub> )	4.3	0.3
18.5	1.6	0.1
19.6	2.1	0.2
22.6	5.7	0.4
23.8	3.4	0.2
24.5	4.1	0.3
24.6	7.5	0.6
25.0(C <sub>25:0</sub> )	30.3	2.3

a Equivalent chain length

b Fatty acids < 1.0% not listed

C where x is carbon number

x:y

y is number of unsaturated bonds

### 3.2.4.5. GLC analysis of cuticular triglycerides.

Fractional equivalent chain length values suggested that some triglyceride fatty acids were unsaturated (Table 14). Palmitic acid (16 carbons) was the most abundant fatty acid (33.1%) followed by oleic acid (18 carbons, mono-unsaturated, 20.6%) and a fatty acid thought to be unsaturated and contain 23 carbons (ECL 22.6, 12.6%).

### 3.2.4.6. GLC analysis of cuticular fatty acids.

Palmitic acid (16 carbons) and stearic acid (18 carbons) at 26.1% and 18.2% respectively were found to be the major fatty acids. Fractional equivalent chain lengths for many of the minor components indicated the likelihood of their being unsaturated. The fatty acids comprise the least abundant class of the cuticular lipids (3.87% by weight), hence the overall concentration of the predominant fatty acid (palmitic acid) is extremely low (approximately 1%). The main fatty acids are listed in Table 15. Many minor fatty acids were present (i.e. less than 2%); however, their levels of concentration in the total wax might be regarded as insignificant (i.e. less than 0.08%).

### 3.2.4.7. GLC analysis of cuticular 1, 3 - diglycerides.

Palmitic acid (17.6%) was found to be the most abundant diglyceride fatty acid. Table 16 shows many of the fatty acids possessed fractional equivalent chain length values, due possibly to a high occurrence of unsaturation amongst the fatty acid chains.

Table 14

Fatty acid composition of cuticular  
triglycerides

Fatty acid E.C.L. <sup>a</sup>	Mean % Composition	
	of total triglyceride <sup>b</sup> fatty acids	of wax
14.0(C <sub>14:0</sub> )	1.8	0.1
15.6	1.9	0.1
16.0(C <sub>16:0</sub> )	33.1	1.9
17.8(C <sub>18:1</sub> )	20.8	1.2
18.0(C <sub>18:0</sub> )	7.6	0.4
18.5	2.3	0.1
19.6	3.5	0.2
20.5	1.4	0.1
22.2	3.5	0.2
22.6	12.6	0.7

<sup>a</sup> Equivalent chain length

<sup>b</sup> Fatty acids < 1.0% not listed

C<sub>x:y</sub> where x is carbon number

y is number of unsaturated bonds

Table 15

## Composition of cuticular fatty acids

Fatty acid E.C.L. <sup>a</sup>	Mean % Composition	
	of total fatty acids <sup>b</sup>	of wax
14.0 (C <sub>14:0</sub> )	2.5	0.1
16.0 (C <sub>16:0</sub> )	26.1	1.0
17.6 (C <sub>18:1</sub> )	5.0	0.2
18.0 (C <sub>18:0</sub> )	18.2	0.7
19.6	7.8	0.3
20.0 (C <sub>20:0</sub> )	2.4	0.1

a. equivalent chain length

b. fatty acids  $\leq$  2.0 % not listed

Table 16

Fatty acid composition of cuticular 1, 3-diglycerides

Fatty acid E.C.L. <sup>a</sup>	Mean % Composition	
	of total <sup>b</sup> diglyceride fatty acids	of wax
13.8	2.4	0.1
14.0(C <sub>14:0</sub> )	2.0	0.1
14.8	6.4	0.3
15.8	8.9	0.4
16.0(C <sub>16:0</sub> )	17.6	0.8
16.8	8.3	0.4
17.9	7.6	0.3
18.0(C <sub>18:0</sub> )	8.4	0.4
18.9	10.5	0.5
19.8	10.0	0.5
20.8	7.7	0.4
21.8	4.9	0.2

<sup>a</sup> Equivalent chain length<sup>b</sup> Fatty acids  $\leq$  2.0% not listed.

### 3.2.5. Examination of synthesis product (15-hentriacontene).

The following products were prepared as intermediate reagents for the synthesis of 15 - hentriacontene (see section 2.2.5. for synthesis chart).

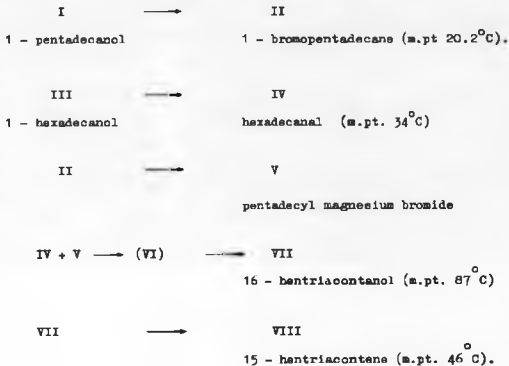


Fig. 23 shows the chromatogram obtained when the synthesis end-product (VIII) was analysed by GLC. The impurities were minor by comparison with the major peak (VIII) and appeared to consist of straight alkanes and mono-unsaturated alkenes.

The mass spectrum (Fig. 24 ) of the major peak was identical with that from peak number 27 (see section 2.2.4.7.). Both



spectra contained molecular ions at  $m/e$  434 and <sup>similar</sup> fragment ion patterns. Ions were observed at  $m/e$  55/57, 69/71, 83, 97, 111, 125, 139, 153, 167, 181, 195 etc. (at  $m/e$  14 intervals) and the relative abundance of each ion in one spectrum corresponded with that of its counterpart in the other. Mass spectroscopic examination also revealed that the major GC peak attributed to the synthesis product comprised two unresolved peaks each possessing molecular ions at  $m/e$  434. These peaks corresponded to the cis - and trans - isomers of the alkene, 15 - hentriacontene.

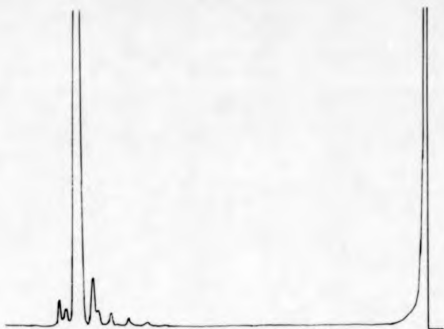


Figure 23. Gas chromatogram of synthesised product, 15-hentriacontene.

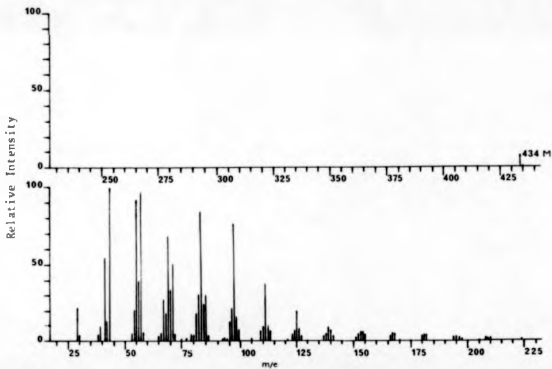


Figure 24. Mass spectrum of synthesised product, 15-hentriacontene.

### 3.3. Applied aspects

#### 3.3.1. Physical characteristics of cuticular lipids and adult lice

##### 3.3.1.1. Melting-point determination of cuticular wax.

When subjected to a slow temperature increase, cuticular louse wax began softening around 41-42°C (Table 17) and gave the appearance that the melting was restricted to a minor component of the material. The condition remained constant until the temperature reached 49.5 - 50.5°C (Table 17) when the solid wax, which formed at least 90% of the total, melted sharply. By allowing the waxes to cool in the melting-point apparatus, the temperatures of solidification were observed and found to coincide with the corresponding melting-point temperatures.

Table 17

Melting-point temperatures of cuticular waxes

Wax Sample	Temperature of initial melting	Temperature of complete melting
1	42 °C	50.0 - 51.0
2	41 °C	49.0 - 50.5
3	42 °C	49.5 - 50.5

3.3.1.2. Solubility of simulated cuticular wax in potential insecticide solvents.

The synthetic cuticular wax dissolved readily in potential insecticide solvents, didecyl ether, 1 - octadecene and n - pentadecane giving concentrations greater than 1.0 g/100 ml of solvent (Table 18 ). Solutions of the wax were less readily formed in dodecanal, dodecanol, 2 - dodecanone and undecanol and the maximum possible concentrations of the wax fell in the range 0.5 - 1.0 g/100 ml of solvent. Lauryl ether dissolved the wax at concentrations not exceeding 0.25 g/100 ml of solvent while 1, 4 - butanediol and 1, 2 - propanediol failed to dissolve the wax to any appreciable extent.

3.3.1.3. Determination of upper lethal temperature of adult lice.

When subjected to an increase in temperature, both male and female lice died in the 50 - 52°C range (Table 19 ) after a layer of moisture had been noted on the cuticular surface of the insects at temperature immediately prior to the thermal death-point.

A record of changes in electrical conductivity on the surface of the adult female louse with temperature increase is shown in Fig. 25. The vibrational effect in the trace was caused by insect movement and increased dramatically above 46°C.

Table 18

Solubility of synthetic cuticular wax in  
potential insecticide solvents

Potential insecticide solvent	Solubility of 1 mg wax in insecticide solvent			
	a 100 $\mu$ l solvent	b 200 $\mu$ l solvent	c 300 $\mu$ l solvent	d 400 $\mu$ l solvent
1, 4 - butanediol	0	0	0	0
diisocyl/ether	+	+	+	+
dodecanal	0	+	+	+
dodecanol	0	+	+	+
2 - dodecanone	0	+	+	+
lauryl ether	0	0	0	+
1 - octadecene	+	+	+	+
n - pentadecane	+	+	+	+
1, 2 - propanediol	0	0	0	0
undecanol	0	+	+	+

0 - insoluble

+

soluble

a - concentration 1.0g wax/100 ml. solvent

b - concentration 0.5g wax/100 ml. solvent

c - concentration 0.33g wax/100 ml. solvent

d - concentration 0.25g wax/100 ml. solvent

Table 19

Thermal death-points of adult lice

Louse number	Males Thermal death-point °C	Females Thermal death-point °C
1	52	51
2	52	51
3	51	50
4	50	52
5	51	51
6	50	52
7	50	52
8	52	51
9	50	52
10	51	51
Average	50.9	51.3

## Recorder Response

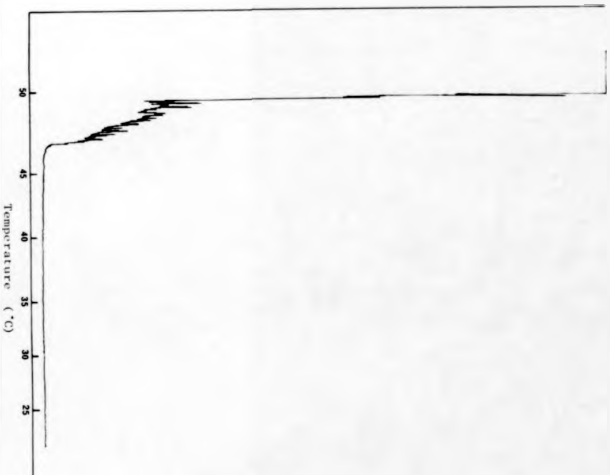


Figure 25. Recorder trace of change in electrical conductivity on the surface of a female adult louse with temperature increase.

It was not possible to conduct the experiments with adult male lice since moisture appeared to cover the insects within moments of their being placed in the melting-point tubes thus allowing the passage of electrical current between the wires of the electrode before any increase in temperature.

The traces produced by the female adults (Fig. 25 ) possessed relatively steady baselines as the temperature was slowly raised to  $46 - 47^{\circ}\text{C}$ . A marked increase in the recorder response was observed after reaching this temperature range and was followed by a more dramatic increase on reaching  $48 - 49^{\circ}\text{C}$ . Death of the insect occurred immediately after (i.e.  $50 - 52^{\circ}\text{C}$ ).

#### 3.3.1.4. Comparative volatilities of potential insecticide solvents.

1, 2 - Propanediol was found to be the most volatile of the potential insecticide solvents (Table 20 ) followed by n - pentadecane which evaporated at a rate 1.4 times slower than that of the diol. With the exceptions of didecyl ether and lauryl ether, which proved to be virtually non-volatile, the remaining solvents showed similar evaporation rates and were significantly less volatile than 1, 2 - propanediol and n - pentadecane (Table 20 ).



Table 20  
Comparison of times for complete  
evaporation of (5  $\mu$ l) potential  
insecticide solvents (at 30°C and  
75% R.H.)

Potential insecticide solvent	Average evaporation rate (30°C / 75% RH) $\mu$ l/hour
1, 2 - propanediol	0.250 $\pm$ 0.012
n - pentadecane	0.178 $\pm$ 0.006
2 - dodecanone	0.052 $\pm$ 0.002
dodecanal	0.035 $\pm$ 0.001
1, 4 - butanediol	0.030 $\pm$ 0.001
undecanol	0.026 $\pm$ 0.001
1 - octadecene	0.026 $\pm$ 0.001
dodecanol	0.023 $\pm$ 0.001
didecyl ether	non volatile
lauryl ether	non volatile

### 3.3.2. Toxicity tests with adult lice.

#### 3.3.2.1. Protocol for toxicity tests.

The mortality rates produced by three solvents (dodecanol, 2, - dodecanone and undecanol) among the adult female lice were markedly affected by the type of container in which the lice were separately held for the 24 hour test period (Table 21 ). For each solvent, the dosage applied per louse was more than sufficient to cause a mortality rate of 100% (see section 3.3.2.2.). Only toxicity tests carried out using glass containers consistently gave mortality rates of 100%, whereas the rates obtained with the plastic containers were significantly lower for all solvents. The most conspicuous disparity between results was that observed for 2 - dodecanone (Table 21) where 64  $\mu$ g of solvent/ louse killed 100% of lice held in glass containers, 78.6% of lice held in plastic containers (A) and 3.3% of lice held in plastic containers (B).

Fig. 26 shows the regression lines produced by computer analysis of data from lauryl ether toxicity tests in which glass containers and plastic containers (B) had been used. Results with the plastic containers compared to those with the glass (Table 22 ) indicated that it was necessary to apply 12 times more lauryl ether per insect in order to achieve 50% mortality ( $LD_{50}$ ) and 16 times more for 90% mortality ( $LD_{90}$ ).

Table 21

Comparison of mortalities amongst adult female  
lice held in different container types after  
topical application

Test solvent	Container	Solvent dose $\mu\text{g}/\text{louse}$	Mean <sup>a</sup> mortality %	Standard deviation %
dodecanol	glass	120	100.0	$\pm$ 0.0
	plastic A	120	66.6	$\pm$ 3.0
	plastic B	120	44.5	$\pm$ 2.5
2-dodecanone	glass	64	100.0	$\pm$ 0.0
	plastic A	64	78.6	$\pm$ 6.6
	plastic B	64	3.3	$\pm$ 1.5
undecanol	glass	66	100.0	$\pm$ 0.0
	plastic A	66	68.3	$\pm$ 4.9
	plastic B	66	41.9	$\pm$ 7.7

<sup>a</sup> - mean mortalities of 3 replicates

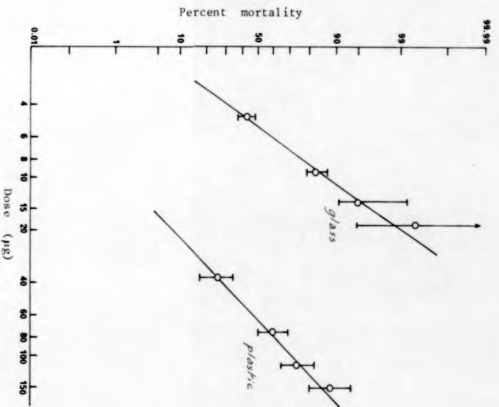


Figure 26. Comparison of lauryl ether toxicities using glass and plastic containers.

Test solvent	LD <sub>50</sub> µg/louse	95% fiducial limits for LD <sub>50</sub>	LD <sub>90</sub> µg/louse	95% fiducial limits for LD <sub>90</sub>	slope (±) standard error	P
Lauryl ether (glass containers)	5.3	5.7 4.9	11.1	12.3 10.3	4.0301 ± 0.2789	> 0.05
Lauryl ether (plastic containers B)	63.7	71.3 55.7	179.8	231.5 151.1	2.8422 ± 0.3128	> 0.05

Table 22

Comparison of results from toxicity tests with lauryl ether  
in which lice were held in glass or plastic (B) containers

Fig. 27 to 36 illustrate the relationships between the solvent masses deposited during topical application and the corresponding number of micrometer divisions used. Linear graphs were obtained for each solvent.

### 3.3.2.2. Topical application of potential insecticide solvents.

Computer analysed dose/mortality data from the toxicity tests with the potential insecticide solvents are shown in Table 23 and the results were also used to construct the individual regression lines for the solvents on probit-log graph paper (Fig. 37 to 46).

Comparing toxicities of the solvents on the basis of their  $LD_{50}$  and  $LD_{25}$  levels, lauryl ether (5.3  $\mu\text{g/louse}$  and 11.1  $\mu\text{g/louse}$  respectively) was found to be the most potent compound (Table 23) followed by dodecanol (7.2  $\mu\text{g/louse}$  and 13.7  $\mu\text{g/louse}$  respectively). An examination of the collective regression lines of the 10 solvents (Fig. 47) revealed that 3 broad groups were formed according to toxicity; with lauryl ether, dodecanol, 2 - dodecanone and undecanol constituting the most toxic group. A second group, 3 to 4 times less toxic, was formed by 1 - octadecene, dodecanal, n - pentadecane and didecyl ether while a third group which was not appreciably toxic was formed by 1, 2-propanediol and 1, 4-butanediol.

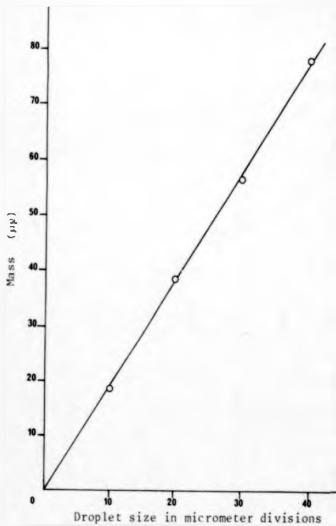


Figure 27. Plot of micrometer reading vs droplet mass for 1,4-butanediol.

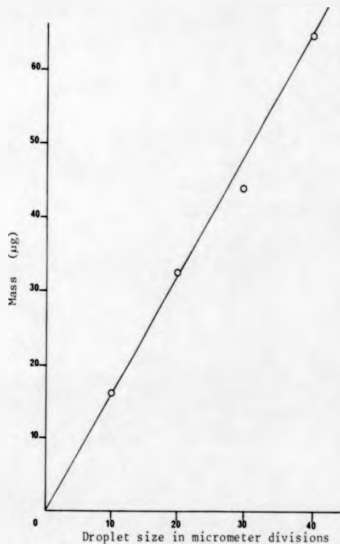


Figure 28. Plot of micrometer reading vs droplet mass for didecyl ether.

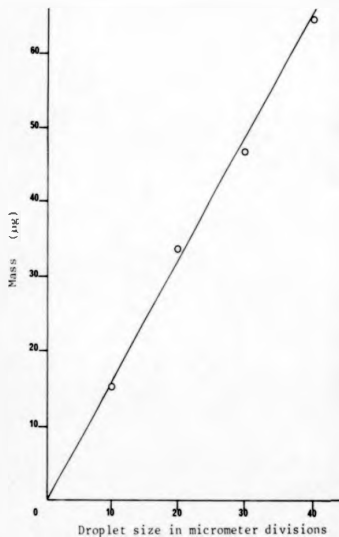


Figure 29. Plot of micrometer reading vs droplet mass for dodecanal.

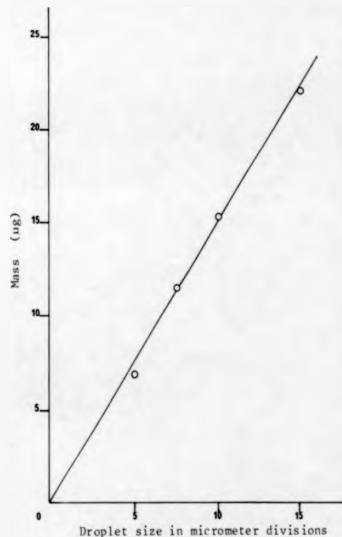


Figure 30. Plot of micrometer reading vs droplet mass for dodecanol.



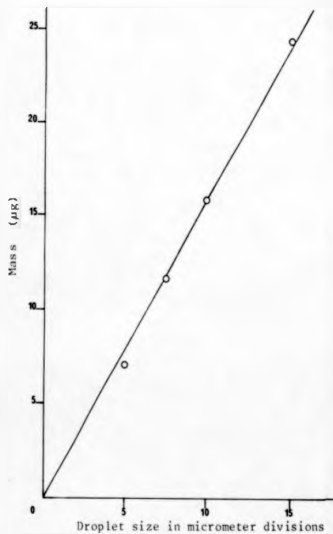


Figure 31. Plot of micrometer reading vs droplet mass for 2-dodecanone.

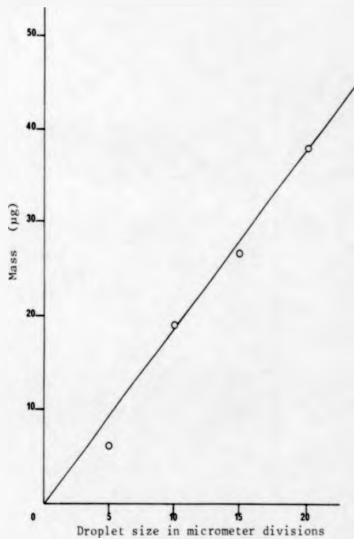


Figure 32. Plot of micrometer reading vs droplet mass for lauryl ether.

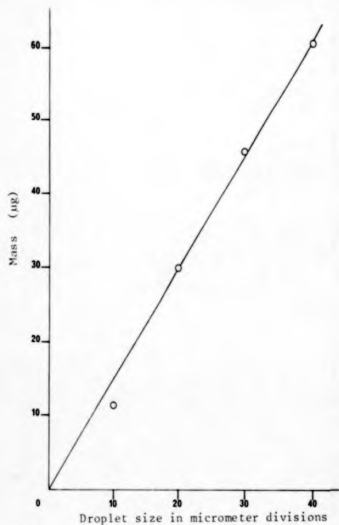


Figure 33. Plot of micrometer reading vs droplet mass for 1-octadecene.

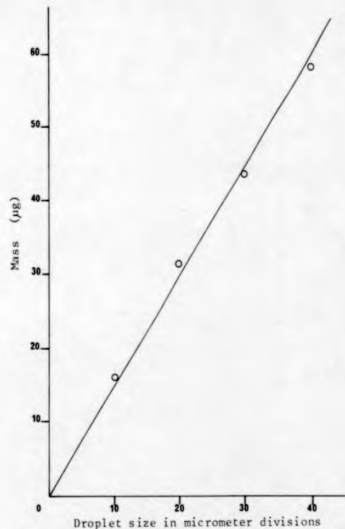


Figure 34. Plot of micrometer reading vs droplet mass for n-pentadecane.

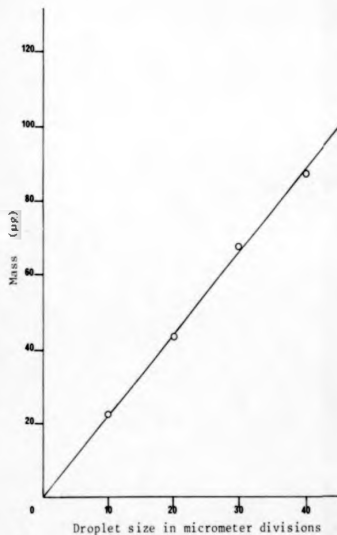


Figure 35. Plot of micrometer reading vs droplet mass for 1,2-propanediol.

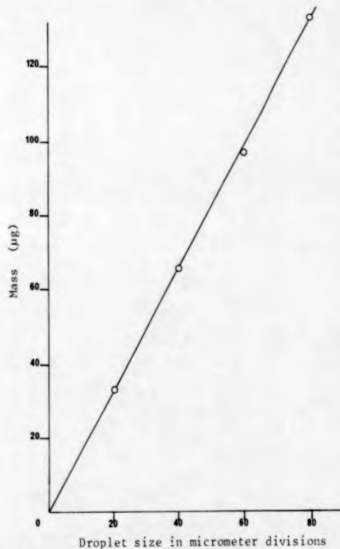


Figure 36. Plot of micrometer reading vs droplet mass for undecanol.

Table 3

Test solvent	$\frac{LD_{50}}{\mu\text{g/1000g}}$	95% fiducial limits for $\frac{LD_{50}}{\mu\text{g/1000g}}$	$\frac{LD_{50}}{\mu\text{g/1000g}}$	95% fiducial limits for $\frac{LD_{50}}{\mu\text{g/1000g}}$	Slope (g) Standard error	P
1,2 - dichloroethane	—	—	—	—	0.6267 ± 0.2956	0.05
1,2-dichloro ether	78.3	33.0 12.9	63.8	44.5 60.1	4.7660 ± 0.2331	0.05
Acetone	27.2	24.3 25.4	69.9	79.2 63.2	3.129 ± 0.1962	0.08
Acetone	3.3	7.6 4.9	13.7	14.7 12.4	4.6491 ± 0.2040	0.05
1,2 - dichloroethane	10.0	10.5 5.7	14.5	15.2 13.9	8.0527 ± 0.4503	0.05
1,2-dichloro ether	5.5	5.7 4.9	11.1	12.2 10.3	4.0301 ± 0.2799	0.05
1 - trichloroethane	74.2	25.7 27.8	95.6	41.4 51.2	5.5561 ± 0.2064	0.05
n - pentadecane	29.6	51.2 27.9	73.1	79.4 66.2	3.3667 ± 0.4488	0.05
1,2 - dichloroethane	—	—	—	—	1.064 ± 0.347	0.05
Acetone	11.0	11.1 10.6	17.5	19.5 16.7	6.275 ± 0.3410	0.05

Results of toxicity tests using solvent and/or test oil (purity 100%)

Source: U.S. Department of Agriculture, Bureau of Entomology and Plant Quarantine

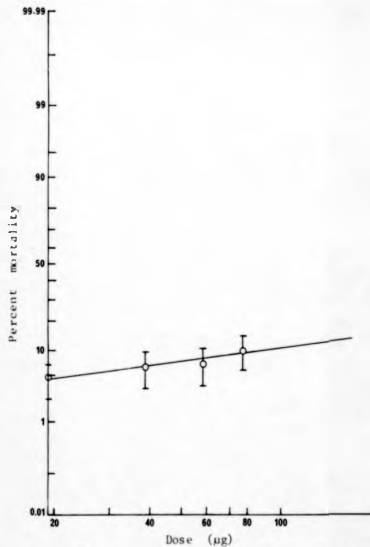


Figure 37. Regression line for 1,4-butanediol

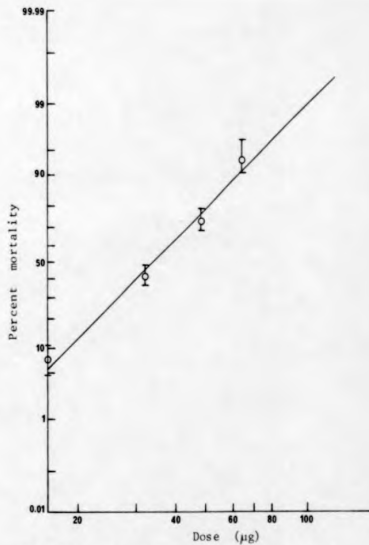
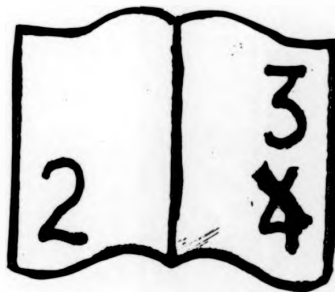


Figure 38. Regression line for didecyl ether.

# PAGINATION ERROR

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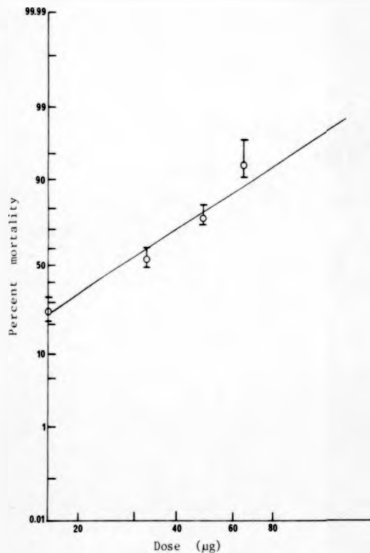


Figure 39. Regression line for dodecanol.

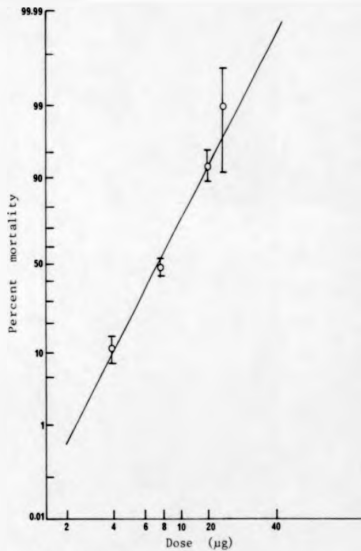


Figure 40. Regression line for dodecanol.

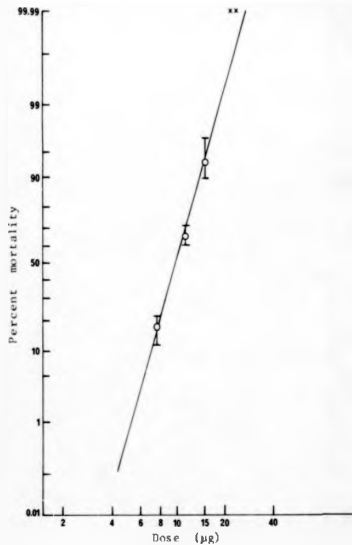


Figure 41. Regression line for 2-dodecanone.

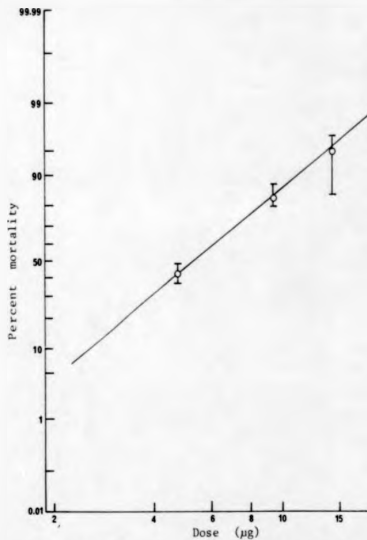


Figure 42. Regression line for lauryl ether.



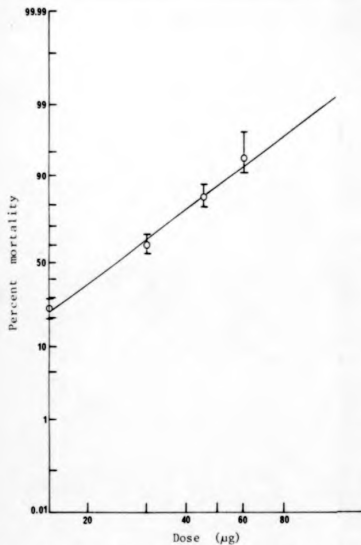


Figure 43. Regression line for n-octadecene.

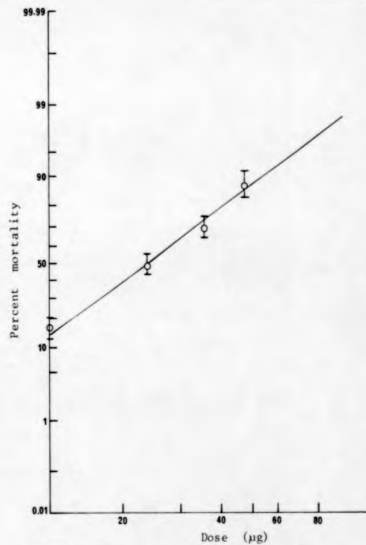


Figure 44. Regression line for n-pentadecane.

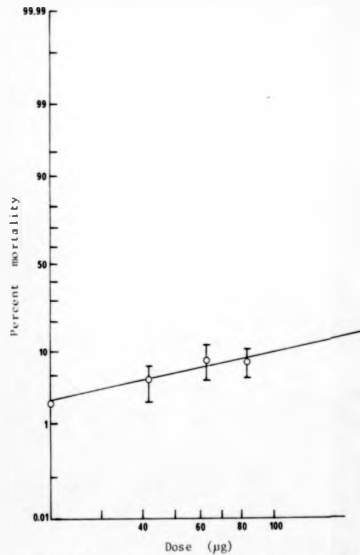


Figure 45. Regression line for 1,2-propanediol.

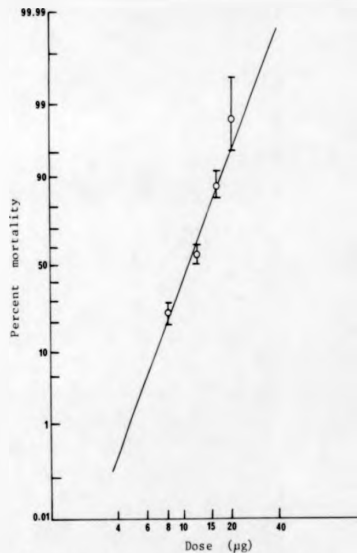


Figure 46. Regression line for undecanol.

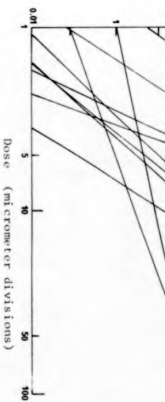
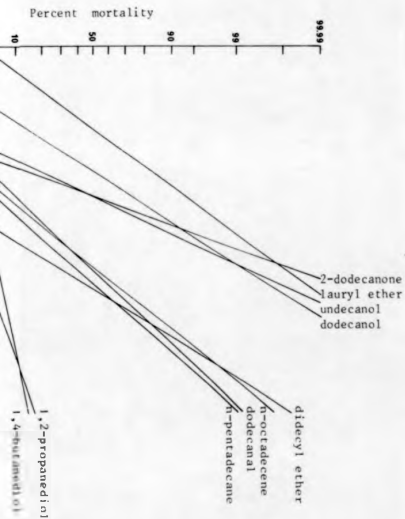


Figure 47. Combined regression lines for the potential insecticide solvents.



3.3.2.3. Topical application of aqueous insecticide solvent mixture.

The 10% (v/v) mixture of lauryl ether in distilled water formed a light gel from which the water readily evaporated if left uncovered. Consequently, it was impossible to determine the mass of the topically applied droplets with the Cahn Electrobalance as described (see section 2.3.3.3.). However, when weighing droplets of the ten potential insecticide solvents (see section 2.3.3.3.), it was found that, on average 95.3% of the volume shown was actually deposited during topical application. Values for the  $LD_{50}$  and  $LD_{90}$  were adjusted accordingly after being calculated as described (see section 2.3.3.2.). The regression line for the 10% lauryl ether mixture (Fig. 48) was plotted as described (see section 2.3.3.2.) while a second graph (Fig. 49) was constructed which compared the lines produced by lauryl ether in the pure form and that actually present in the 10% mixture.

While there was close general agreement between the 2 regression lines (e.g. coincident  $LD_{90}$  values), a small difference in slopes was observed which resulted in 18.9% more lauryl ether being necessary to achieve 50% mortality when the solvent was used in 10% aqueous mixture rather than the pure form.

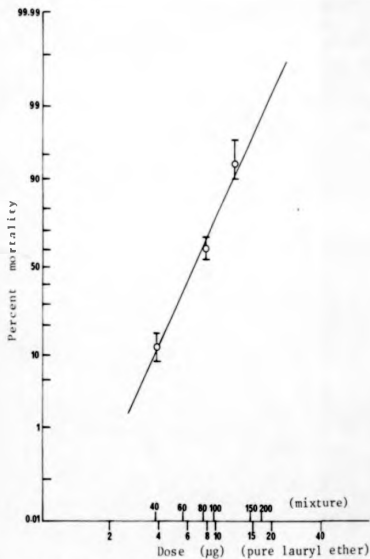


Figure 48. Toxicity of 10% aqueous lauryl ether (gel).

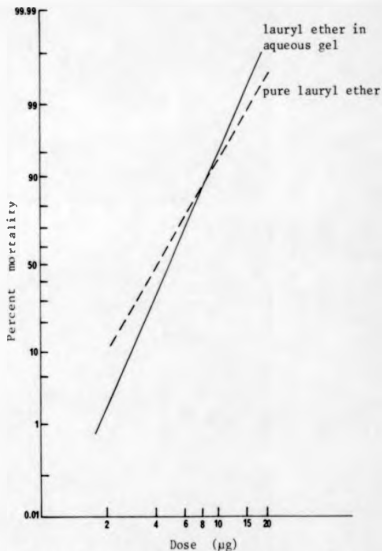


Figure 49. Comparison of lauryl ether toxicity from pure and aqueous forms.

Test solvent	LD <sub>50</sub> µg/louse	95% fiducial limits for LD <sub>50</sub>	LD <sub>90</sub> µg/louse	95% fiducial limits for LD <sub>90</sub>
10% (v/v) lauryl ether in water	67.1	70.1 64.2	117.1	127.3 109.3
lauryl ether (present (in 10% mixture)	6.3	6.6 6.1	11.1	12.2 10.3
lauryl ether (applied as pure)	5.3	5.7 4.9	11.1	12.2 10.3

Table 24

Comparison of lauryl ether toxicity resulting from  
pure and aqueous forms



3.3.2.4. Topical application of insecticide (dieldrin)  
in potential insecticide solvent.

After topical application of lice with doses of 0.005  $\mu$ l/louse, lauryl ether produced an average rate of mortality of 42.6% while 1, 4 - butanediol applied at the same dosage produced no mortalities (Table 25). The average mortality rate increased to 70.6% and 100% when the same volume of lauryl ether per louse (0.005  $\mu$ l) contained 0.02  $\mu$ g of dieldrin and 0.2  $\mu$ g of dieldrin respectively. A similar addition of dieldrin to 1, 4 - butanediol resulting in 0.02  $\mu$ g and 0.2  $\mu$ g of dieldrin in 0.005  $\mu$ l of solvent per louse produced mortality rates of 12.3% and 100% respectively.

Therefore, the increase in mortality attributed to the dieldrin (0.02  $\mu$ g/louse) in the 1, 4 - butanediol was 12.3% whilst that attributed to the dieldrin at the same level in the lauryl ether was 28%.

Table 25

Comparison of dieldrin toxicity resulting from solutions in different insecticide solvents

Insecticide solvent	solvent volume per louse (μl)	Dieldrin per louse (μg)	% Mortality <sup>a</sup>
1,4-butanediol	0.005	0.00	0.0
	0.005	0.02	12.3
	0.005	0.20	100.0
lauryl ether	0.005	0.00	42.6
	0.005	0.02	70.6
	0.005	0.20	100.0

a - average of 3 replicates

3.3.3. Observation of cuticular penetration of potential insecticide solvents using fluorescent dyes (Rhodamine B).

Cryostat sections from untreated lice and lice treated with solvents exclusive of Rhodamine B (lauryl ether, dodecanol and 1, 4 - butanediol) displayed no significant fluorescence when examined under fluorescence microscopy with a Rhodamine system (Plates 48 and 49). Consistent results were produced irrespective of treatment periods in the case of the solvent treated lice.

Lice treated with either lauryl ether or dodecanol were visibly affected after 30 minutes; but examination for Rhodamine fluorescence in sections from lice topically applied with the solutions of Rhodamine B, did not reveal any noticeable increases in fluorescence. After 60 minutes, when most of these lice had died, fluorescence was observed in sections of cuticle containing some degree of sclerotisation (Plates 50 and 52). The effects were more pronounced in sections taken from lice four hours after the initial treatment with either the lauryl ether or dodecanol Rhodamine B solutions (Plates 51, 53, 54 and 55).

In contrast with unsclerotised cuticle, sclerotised cuticle was distinguished by a red-orange fluorescence (yellow-orange in micrographs) after the four hour period described above;

Plate 48. Untreated sclerotized cuticle from paratergal plate (near spiracle). No auto fluorescence is evident in the Rhodamine B region. x 1000.

Plate 49. Cuticle at the junction of tergum (membranous) and paratergite (sclerotized) of female adult treated with lauryl ether only (sectioned after 4 hours). No Rhodamine fluorescence is observed. x 1000.

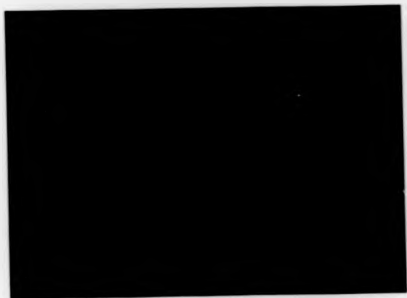
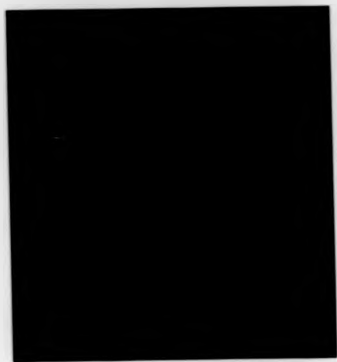


Plate 50. Postnotus (membranous) separating subcoxae (sclerite) of female adult louse (dead) 1 hour after treatment with lauryl ether/Rhodamine B. Faint fluorescence is evident in sclerotised cuticle. x 1000.

Plate 51. Cuticle from same location as in Plate 50 but sectioned 4 hours after treatment with lauryl ether/Rhodamine B. Fluorescence in sclerotised cuticle is stronger and pore canals are visible as striations. x 1000.

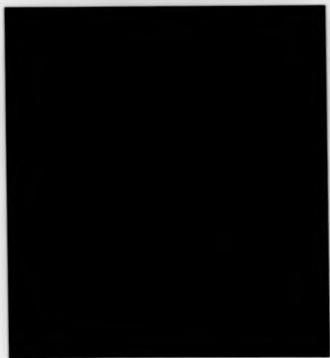


Plate 52. Cuticle from junction of rear leg and coxa of female adult louse (dead) 1 hour after treatment with lauryl ether/Rhodamine B. Fluorescence is observed in sclerotised cuticle. x 1000.

Plate 53. Cuticle from dorsal metathoracic subcoxa (above leg joint) of male adult louse sectioned 4 hours after dodecanol/Rhodamine B treatment. Fluorescence is evident throughout cuticle (sclerite) and tracheas (arrows). x 1000.



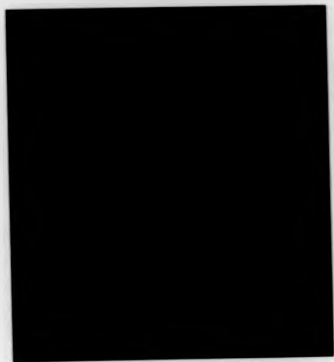
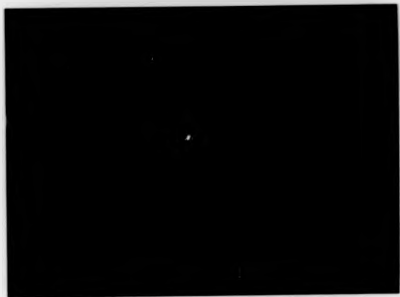


Plate 54. Cuticle from junction of leg and coxa of female adult louse sectioned 4 hours after treatment with lauryl ether/Rhodamine B. Fluorescence is observed mainly in sclerotised cuticle but also to a lesser degree in unsclerotised rugose cuticle. x 1000.

Plate 55. Thoracic cuticle from ventral surface of female adult louse sectioned 4 hours after dcdscanol/Rhodamine B treatment. Fluorescence is seen throughout the cuticle (sclerite) and pore canals are visible as striations. x 1000.



examples of which were found in transverse sections showing the dorsal region of the thorax (Plates 51 and 53), the thorax-abdomen junction (Plate 54), the ventral region of the thorax (Plate 55) and the perimeters of the paratergal plates. Rugose cuticle and rubber-like cuticle were not stained by the Rhodamine B (lauryl ether, dodecanol) solutions to the same degree as sclerotised cuticle despite acting, in many instances, as an effective junction between two sclerotised regions (Plates 51, 53 and 54).

In cuticle which was particularly well stained with the dye (Plate 55), pore canals were distinctly visible as fluorescent, vertical striations in the cuticular cross section. Fluorescence was also observed in the trachea of insects dosed with Rhodamine B/ dodecanol solution (Plate 53) which provided evidence that entry of the insect by the solution had been gained through the spiracles and the tracheal system.

Lice applied with 1, 4 - butanediol (either pure or solution containing Rhodamine B) produced no mortalities during the tests. Under UV examination, sections of the lice dosed with the Rhodamine B/ 1, 4 - butanediol solution taken 30 minutes and 60 minutes after treatment exhibited no significant increases in fluorescence. A fluorescent layer covering the cuticle was observed, however, in sections taken from lice four hours after the initial treatment (Plate 56).

Plate 56. Cuticle from junction of tergum and paratergite of female adult louse 4 hours after treatment with 1,4-butanediol/Rhodamine B. Fluorescence is observed on the surface only. X 1000.



#### 4. Discussion

##### 4.1. Physical structure.

##### 4.1.1. External features.

The cuticles of some arthropods are capable of rapid expansion at some stage in their development and examples of such cuticles may be found in caterpillars, Rhodnius prolixus nymphs, queen termites, honey ants and female ticks (Neville, 1975). In each case the expansion occurs in unsclerotised areas of cuticle which is usually folded to some extent. While in the unfed state, Rhodnius prolixus (fifth instar) and Boophilus microplus (adult female) have abdominal surfaces which are deeply folded or rugose (Hackman, 1975) and resemble the membranous abdominal cuticle of the human body louse. Although the Rhodnius nymph and the Boophilus adult feed only once, whereas the louse feeds frequently, the close similarity of their rugose cuticles suggests that some aspects of abdominal expansion in the three animals are similar. The two forms of rugose cuticle found on the body louse are consistent with those previously observed on the abdominal surface of the pubic louse by Ubelaker and co-workers (1973). They described the cuticle on the dorsal abdominal surface of the pubic louse as being rugose, but referred to cuticle on the ventral surface as 'scaled'; a term not wholly correct. The 'scaled' pubic louse cuticle appears exactly the same as the 'quilted' cuticle seen in my studies on the body louse. SEM examinations of the abdominal body louse cuticle revealed that the two distinct rugose cuticle formations were

closely related. Both consisted of corrugations but those of the quilted type were very much shorter in comparison to the long rugosities of the second type found over regions such as the mid-gut where expansion occurs during feeding. TEM examination of both types of rugose cuticle showed them to be structurally identical.

Quilted rugose cuticle was not confined to the abdomen but occurred in other regions such as leg joints, where movement caused by the underlying musculature requires a high degree of flexibility. In fact, an extensive system of muscles connecting the dorsal and ventral areas of quilted rugose cuticle on the abdomen was seen in the stained light microscope sections of whole lice (Plate 9). The series of abdominal pits found on the body louse in the same areas as the quilted rugose cuticle were previously described on the abdominal surface of the pubic louse (Ubelaker *et al.*, 1973) and probably result from attachment of the underlying musculature to the integument. However, I could ascribe no obvious function to the areas of serrated rugose cuticle on the abdomen of the body louse, also noted on the abdomen of the pubic louse (Ubelaker *et al.*, 1973).

Externally, the body louse is distinguished by several other unique features. As in all species of Anoplura, the mouthparts of the body louse are among the most highly specialised found in insects and function by piercing and sucking in a manner quite unlike that of other blood-sucking arthropods (Ferris, 1951). The



claws on the legs are also characteristic in Anoplura and serve as clasping organs which are perfectly suited to an environment containing numerous hairs and fibres etc. (Buxton, 1947). Although eyes are either absent or barely recognisable in most species of Anoplura, a small minority including the body louse possess distinct structures which consist of thickened cuticle (Ferris, 1951). The body louse may also be differentiated from other members of Anoplura by the mushroom-like shape of the spiracles which are characteristic of the family Pediculidae (Ferris, 1935).

#### 4.1.2. The epicuticle.

A review of previous studies shows that some confusion has arisen concerning the terminology used for the various layers comprising the epicuticle. The structure of the epicuticle in sclerite and membranous body louse cuticle closely resembles that described by Filelis and Hadley (1979) in the desert scorpion Hadrurus arizonensis and consequently, I have used this terminology in my study.

Although a wax bloom was apparent in platinum/carbon replicas of cuticle surfaces (Locke, 1974), it was almost completely removed during preparation for TEM examination and only remnants of it remained in well-protected areas. The unsaturated lipids which comprise over 25% of the cuticular wax are readily fixed by osmium tetroxide and this accounts for the appearance in

a few electron micrographs of isolated deposits adjacent to the cuticle surface (Plates 13 and 16). This situation has been reported previously with other arthropods such as the spiny rat mite Laelaps echidnina (Wharton et al., 1968) and the soil mite Oppia colera ensis (Brody, 1970). In fact, Wharton and co-workers demonstrated the presence of wax blooms on the surface opposite pore canals which suggests that cuticular wax traverses the cuticle via the pore canal system in this animal.

In most electron micrographs of louse cuticle the outermost layer of the epicuticle was the outer membrane, which corresponds with the lipid monolayer postulated by Beament (1961, 1964) and apparently demonstrated by Locke (1965, 1966) and Zacharuk (1972). Although the thickness of the layer (about 30 Å) is consistent with that of the cuticular hydrocarbons when aligned at 90° to the surface, I tend to agree with Filshie (1970 a, b) and Lockey (1976) who question the existence of the so called oriented lipid monolayer. Studies with Callosodes (Filshie, 1970a), Lucilia (Filshie, 1970b) and H. arizonensis (Hadley and Filshie, 1979) have shown that in each instance, the outer membrane of the epicuticle was completely resistant to severe extraction under conditions more than sufficient to overcome any forces of attraction which might exist between the monolayer and the epicuticle. Lockey (1976) notes that the non-polar (hydrocarbon) contents of cuticular waxes from P. americana and S. gressaria are essentially equal and comprise the bulk of the waxes (75-77%), which is also the case

with the body louse, and suggests that if the monolayer does exist, it must be a heterogeneous mixture of non-polar and polar lipid molecules. Both Filshie (1970a) and Lockey (1976), however, envisage that the lipid layer would be in a liquid phase to allow replacement of lost wax and if this is so, the lipid monolayer could certainly not survive TEM preparation conditions.

Although the method by which the lipids (or precursors) are transported through the pore canal system is still unclear, there is no doubt that in my studies that the lipids (or precursors) pass through the epicuticle of the louse by way of the epicuticular filaments and are deposited on the surface at the pores or openings. Epicuticular pores are thought to exist in most insects and have been demonstrated in several species including Callosodes (Locke, 1966) and Thermobia (Noble-Hesbitt, 1967) whilst Hadley and Filshie (1979) observed pores in the epicuticle of Hadroporus arizonensis during TEM and SEM studies on the effects of various lipid solvents. The pores, with diameters about 50 times larger than those found on the body louse, were apparent in the SEM only after prolonged treatment using concentrated potassium hydroxide and Hadley and Filshie (1979) suggest that the surface coating might contain mucopolysaccharides<sup>a</sup> which may be digested by strong alkalis. In contrast, I found organic solvents were more effective in removing surface lipids from the louse, whereas treatment with concentrated potassium hydroxide

merely resulted in a redistribution of the hydrocarbon fraction which does not respond to saponification.

The horizontal tubules which exist at the interface of the procuticle and the dense layer of the epicuticle in the louse have been observed in only one other arthropod, the female cattle tick Boophilus microplus (Filshie, 1976) in which they form a single-layered, interconnecting network. Though it was not possible to produce tangential sections of louse cuticle for TEM examination, it is assumed that the tubules seen below the epicuticle in transverse sections represent a similar network to that in this species. Filshie (1976) was unable to associate any function with the tubular network in the tick, but think it is significant that both the tick and the louse are blood-suckers and in both cases the abdominal cuticle surface is folded to allow expansion when feeding (Hackman, 1975). Whilst inextensible, the epicuticle is still flexible enough to unfold during abdominal expansion (Richards, 1951). In studies with Rhodnius nymphs Maddrell (1966) demonstrated that the nervous system was involved in the plasticisation of abdominal cuticle and suggested that the stimuli resulting in cuticle plasticisation were those sensations which accompany feeding. He recognised that a number of sensory systems which respond to different stimuli have already been detected in certain insects. Perhaps the tubular network found in the cuticles of the tick and the louse performs a similar function to that of the stretch receptors which occur in the pharynx of Locusta

migratoria migratorioides (Clark and Langley, 1963). Alternatively, the network might simply behave in a pneumatic manner between the sclerotised epicuticle and the unsclerotised exocuticle to lessen the effects of trauma during unfolding of the epicuticle during expansion.

#### 4.1.3. The exocuticle.

Although Neville (1975) has stated that most exocuticles are helicoidal (lamellate) throughout their thickness, there are several instances where this is not the case. The exocuticle in the soft integuments of Rhodnius prolixus (Wigglesworth, 1970) and the rabbit tick Haemaphysalis leporispalustris (Nathanson, 1967) are non-lamellate and Beadle (1974) has suggested that in the non-lamellate exocuticle of the cattle tick Boophilus microplus, the microfibrils have unidirectional orientation. A similar arrangement exists in the exocuticle of louse membranous cuticle except that the microfibril crystallites have formed a macrofibrillar structure. Neville (1975) has interpreted cuticular divisions on the basis of staining with Mallory's triple stain (Baker, 1958) and concludes that sclerotised exocuticle is refractile to staining whereas unsclerotised exocuticle, which is impregnated with proteins and lipids, usually stains red. These observations are in agreement with those on sclerotised and membranous louse cuticle respectively after exposure to Mallory's triple stain. The brittle failure of the exocuticle during the fracture of membranous cuticle indicates

that the exocuticle is sclerotised; but the fixed condition of the cuticle caused by the osmium tetroxide treatment and critical point drying probably account for this anomalous behaviour. During expansion of the abdominal cuticle brought about by feeding, unfolding of the epicuticle is accompanied by a limited deformation of the exocuticle. Given the inextensible nature of the epicuticle and the alignment of the unidirectional exocuticle macrofibrils parallel to the axes of the rugosities, one would expect stretching of the exocuticle to be perpendicular to the axes of the rugosities with little or no extension along the rugosities. In the membranous cuticle the underlying endocuticle is capable of greater stretching and therefore, the exocuticle may be seen as a transitional layer between two extremes.

#### 4.1.4. The endocuticle.

The different action of Mallory's triple stain on the endocuticle layers in sclerite and membranous leucous cuticle may be interpreted similarly to the exocuticle by applying the rationale of Neville (1975) and Baker (1958). Where large enough spaces occur in the cuticle structure, the larger anions (aniline blue) occupy them to produce a blue stain. In regions of the cuticle where impregnation by proteins and lipids has caused a reduction in the spaces between the molecular chains, only the

smaller anion (acid fuchsin) can lodge which results in a red stain. Where both impregnation and tanning have occurred, neither of the anions can penetrate the matrix and the cuticle does not stain, which is the case with the endocuticle of louse sclerite cuticle. In contrast, the endocuticle in Mallory stained membranous cuticle possessed an overall mauve colouration composed of red and blue. This variable reaction is consistent with the role that the endocuticle plays in the expansion of abdominal cuticle when the louse feeds. The extensibility of the abdominal cuticle results largely from stretching of the endocuticle layer brought about by a change in cohesion between the chitin-protein components (Núñez, 1963). Because the membranous abdominal cuticle is subjected to frequent distention through feeding by the louse, spaces of various dimensions are likely to be formed between the molecular chains in the endocuticle matrix on a random and recurring basis and this might account for the dual staining reaction.

The homogeneous, non-lamellate appearance of the sclerite endocuticle in TEM studies and its mechanical properties of strength and stiffness suggest that it is composed throughout of unidirectionally oriented microfibrils (Neville, 1975) which is quite uncommon. Evidence of a lamellate structure in the endocuticle was revealed in SEM studies of fractured cuticle and this corresponds with the formation of growth layers deposited after blood meals. A similar phenomenon occurs in the tick Ixodes ricinus (Lees, 1952) and the bug Rhodnius (Zwicky and Wigglesworth, 1956).

In membranous louse cuticle the structure of the endocuticle which is composed of helicoidally arranged (Bouligand, 1965) 250 - 1000 Å macrofibrils closely resembles that observed in the chela tendon of the crab Carcinus maenas (Neville, 1975). Interestingly, the original studies by Bouligand (1965), whereby the helicoidal structure in cuticle was recognised, were performed on Crustacean cuticle which is also constructed of macrofibrils. One of the most interesting theories to explain the formation of unidirectional and helicoidal structure in cuticle involves the self-assembly of liquid crystalline material secreted by the epidermal cells (Neville and Caveney, 1969). It has been suggested (Neville, 1975; Wigglesworth, 1976) that a cholesteric component is responsible for controlling the angle of twist of the helicoid and, in fact, compounds such as cholesteryl esters are frequently found in cuticular lipid extracts. Cholesteryl esters occur in louse cuticular lipids and comprise over 7% of the total (by weight).

Although some points of similarity in the membranous abdominal cuticles of the louse, Rhodnius larva and Boophilus adult were discussed earlier, the endocuticle structure in the cuticles of the latter two arthropods is microfibrillar, unlike the macrofibrillar structure in the endocuticle of the louse. In the Rhodnius larva and the Boophilus adult, cuticle thickness is increased by synthesising additional endocuticle prior to feeding so that stretching of the cuticle can take place during abdominal expansion (Hackman, 1975). Since the louse adult feeds much more frequently than the Rhodnius larva and the Boophilus adult, its abdominal



expansion is not particularly dependent upon increased endocuticle deposition before each meal. Expansion of the abdomen is limited by the inextensibility of the chitin macrofibrils.

Electron micrographs revealed that the macrofibril layers in the membranous endocuticle of the louse are folded to some extent which, no doubt, plays an important part in permitting maximum abdominal expansion. Whilst the structural form of the chitin within the abdominal cuticle of the louse differs from that in Rhodnius and Boophilus, the mechanics of endocuticle plasticisation which permits stretching probably follow the same principles in each. All cuticles have a two-phase composite structure of high modulus polysaccharide fibres of chitin distributed in a low modulus matrix of protein. Bonds exist between the molecules of the two phases but in Rhodnius and presumably other blood-feeders, they are sensitive to pH change (Reynolds, 1975). Maddrell (1966) has shown that plasticisation of Rhodnius abdominal cuticle is under nervous control from the head and Neville (1975) has expressed the view that plasticisation is a highly complex event which is caused by a pH change within the cuticle controlled by the nervous system via the epidermis. Disruption of the pH - labile bonds between chitin and protein molecules allows a degree of sliding to occur between the two phases. Hackman (1975) has suggested that the components of flexible cuticles must not be so highly ordered or closely-packed that crystals are formed and thereby cause a gross change in physical properties. In the light of this statement, the results of Rhodamine B staining of louse cuticle (see section 3.3.4.) are interesting. Under identical experimental conditions,

sclerite cuticle was consistently more heavily stained with Rhodamine B than membranous cuticle, indicating that the sclerite cuticle contains a higher number of unconjugated protein bonds. Recently, Vincent and Millerton (1979) suggested that sclerotisation of cuticle results from highly controlled dehydration within the matrix allowing a large number of sites to become available for conjugation with chemicals involved in tanning, or in this instance, the protein dye Rhodamine B. Maddrell (1966) found that an increase in hydration of the cuticle accompanies plasticisation and this might account for the low uptake of Rhodamine B in the louse membranous cuticle which is, in any case, always hydrated to some extent. In fact, Neville (1970) stresses that water is an essential component of cuticle, since dehydration increases the bonding between the chitin and protein molecules which results in brittleness.

#### 4.1.5. The epidermis.

Integument examined during the TEM studies was taken from post-ecdysial adult lice, usually 2-3 days old, and in each case the decrease in activity following ecdysis was reflected in the appearance of the epidermal cells which were thin and elongated. In view of the rough endoplasmic reticulum and free ribosomes within the cytoplasm of the cells and their function of protein production (Bloom and Fawcett, 1975), it appears that endocuticle continues to be deposited until at least 2-3 days after ecdysis.

Although it has been shown in Schistocerca gregaria (Diehl, 1973) that the oenocytes are responsible for the synthesis of cuticular lipids and that these cells have also been implicated in the production of structural lipids in Rhodnius (Wigglesworth, 1970), the epidermal cells in the louse may, perhaps, feature more prominently in lipid production.

Results from the experiments in which adult lice ingested blood labelled with tritiated oleic acid imply that the turnover of cuticular lipids in the louse is rapid and probably dependent to some degree on feeding. In the epidermis the basal extremities of the pore canal filaments appear to maintain a close extracellular relationship with the epidermal cells suggesting that these cells are responsible for synthesising the major proportion of the cuticular lipids (or precursors). A deduction supported by the observation that oenocytes were not immediately evident in the electron micrographs.

#### 4.1.6. The pore canal system.

The TEM and SEM examinations of louse cuticle have established that the surface of the cuticle is linked to the epidermis via the pore canal/epicuticular filament system; also proposed as the pathway for lipids or precursors involved in waterproofing of the epicuticle (Locke, 1961; Gluud, 1968). It has been known for some time that the louse possesses epicuticular pores

(Wigglesworth, 1942) and it has since become evident that the existence of the pore canal system is crucial in the development of the cuticle as a whole. As well as being involved in waterproofing of the epicuticle, the pore canals facilitate impregnation of the cuticular layers (Neville, 1975) particularly during sclerotisation (Wigglesworth, 1975b). During ecdysis the moulting fluid gains access to the inner layers of the old cuticle via the newly formed epicuticular channels (or filaments) and the products of digestion then return by the same route (Wigglesworth, 1975b).

Though the platinum/carbon replicas of freeze-fractured membranous cuticle provided some evidence that the pore canals resemble twisted ribbons (Neville and Luke, 1969), the TEM examinations revealed that the structures are similar to those observed in Boophilus microplus adults (Fleishie, 1976) and in Rhodnius prolixus larvae (Wigglesworth, 1975a); where they were described as being roughly cylindrical in cross section. Locke (1961) demonstrated that an esterase is present in the cuticles of Calpodes and Galleria in regions where wax canal filaments (epicuticular filaments) arise and, consequently, he suggested that the final stage of wax synthesis occurs in these regions and involves this esterase. If cuticular lipid synthesis is completed in the epidermis, one might expect to see uniform osmium tetroxide staining throughout the pore canal system, particularly in the case of the louse which has a fairly high unsaturated lipid content. In fact the heaviest staining in

the pore canal system occurred throughout the filamentous network found mainly in the epicuticle and exocuticle. Staining of the pore canals in the endocuticle appeared significantly lighter and was restricted to the walls of the structures (Wigglesworth, 1975a). This would seem to agree with the theory of Lookes (1961) that lipid synthesis is completed in the pore canals where the wax canal filaments arise. On the other hand, Filshie (1970a, b) found that the filaments in the epicuticle and exocuticle layers of Calpodes ethlius and Lucilia cuprina are resistant to acid hydrolysis and the action of lipid solvents, which suggests that the filaments consist of or contain substantial amounts of stabilised compounds (lipids) rather than being composed entirely of free lipids.

An alternative explanation for the variable osmium staining in the pore canal system might also apply. All specimens of cuticle were prepared from unfed lice and if cuticular lipids are produced in response to feeding, we might well expect to observe some visible evidence within the pore canals of the cessation of lipid transport.

The role of the pore canal filaments in the active transport of lipids or precursors to the surface of the cuticle could not be determined from these studies, but in view of their well-defined physical structure noted in the SEM, it does seem likely that they serve to keep the pore canals aligned during expansion and contraction of the membranous cuticle (Wigglesworth, 1975a).

#### 4.2. Cuticular lipids of the body louse.

The extremely low level of radioactive contamination in the cuticular lipid extract from lice fed on labelled blood (see section 2.2.2.3.) demonstrates the ability of the polar solvent, hexane, to extract only a minimum of internal lipids while removing most of the surface lipids. Observations on feeding lice have shown that a small volume of clear liquid is usually discharged from the anus shortly after commencement of blood-feeding and this, rather than the extraction of internal lipids might account for most of the contamination observed.

The total extractable cuticular wax from either sex of the louse, expressed as a percentage of total fresh body weight (0.016 - 0.019%), ranks among the lowest reported for arthropods. Curiously, similar levels have been observed in scorpions such as Paruroctonus mesaensis (0.03 - 0.09%) (Hadley and Jackson, 1977) and Hadrurus arizonensis (0.05%) (Toolson and Hadley, 1977) which are noted for their extremely low water loss rates and the desert tenebrionid beetles Centronotera muricata (0.03%), C. variolosa (0.016%) and Palaeophorus adversus (0.02%) (Hadley, 1978). In the light of studies with the scorpions H. arizonensis and Uroctonus auacheanus, Toolson and Hadley (1977) concluded that the highly impermeable nature of the scorpion cuticle resulted from surface lipid composition rather than the presence of an uncommonly thick layer of lipids. Studies with other species of arthropods indicate that the quantity of wax deposited on the

cuticle surface varies with environmental conditions. Deposits of cuticular hydrocarbons on pupal and post-ecdyial adult flesh-flies Sarcophaga bullata greatly exceed those on larvae up to the third instar which live in a moist environment and prevention of pupariation through contact with water is accompanied by a decrease in the quantity of hydrocarbon synthesised (Arnold and Ragnier, 1975). Cuticular hydrocarbons on adult desert tenebrionid beetles Eleodes arata collected in summer months are almost three times as abundant as those from adults collected during winter and winter beetles which were subjected to a higher temperature ( $35^{\circ}\text{C}$ ) for periods of five and ten weeks showed significant increases in the levels of cuticular hydrocarbons (Hadley, 1977). Arnold et al. (1969) observed that the aquatic naiad form of the big stonefly Pteronarcys californica exhibited a quantity of surface wax which was half that of the terrestrial adult form. This is of particular interest in the present study, since the amount of extractable surface wax on the louse is similar to that found on the naiad big stonefly (0.0%). The suggestion that as the naiad big stonefly inhabits an aquatic environment it requires only minimal amounts of surface lipid to prevent dessication (Arnold et al. 1969), is also appropriate in the case of the louse. Transcuticular water losses for the lice used in my studies have been minimised by maintaining a high humidity (75% relative humidity at  $30^{\circ}\text{C}$ ) during rearing, and ensuring that the insects were fully fed once each day (Beament, 1976). The low amount of surface wax on the louse is, therefore, in keeping with its low requirement

for water conservation.

Hydrocarbons often constitute the most abundant lipid class in the surface wax of arthropods (Jackson and Blomquist, 1976). The cuticular hydrocarbons of the louse are typical in this respect and the percentage (about 74%) of the total surface lipids which the hydrocarbons comprise finds closest agreement with the values from the locust Schistocerca gregaria (76%) (Lockey, 1976) and the fire ants Solenopsis invicta (65-75%) and S. richteri (65-75%) (Lok et al, 1975). These classes which make up the cuticular hydrocarbons of the louse; n - alkanes, n - alkenes, internally branched monomethylalkanes and dimethylalkanes have not been found in this particular combination in other insect species (Lockey, 1980a). n - Alkane content of the louse cuticular hydrocarbons (51.6%) is among the higher levels reported for insects whilst the predominance of the odd-numbered n - alkanes; C<sub>25</sub>, C<sub>27</sub> and C<sub>29</sub>, is common in insect hydrocarbon mixtures. The n - alkane with the longest chain-length contains 33 carbon atoms which is in line with the theory that insects are unable to synthesise n - alkanes larger than 33-35 carbons (Jackson and Blomquist, 1976).

In comparison with the n - alkane fraction in cuticular lipids of other insect species, the n - alkenes of the louse comprise a reasonably high percentage (26.4%) of the total cuticular hydrocarbons, but are not as abundant as those of the male cockroaches



Periplaneta australasiae, P. fuliginosa (Jackson, 1970) and P. japonica (Jackson, 1972). The composition of the louse n - alkene series is typical of that observed in other insects possessing cuticular n - alkenes in that odd-numbered alkenes are predominant and one or two constituents of the series are much more abundant than others. 15 - Hentriacontene, the major n - alkene of the louse, has also been identified in an isomeric mixture in the cuticular hydrocarbons of the tenebrionid beetle Tenebrio molitor (Lockey, 1978a) whilst T. obscurus contains 9 - and 11 - hentriacontenes (Lockey, 1978b). A hentriacontene (probably 9 - hentriacontene) was detected as a minor cuticular hydrocarbon component in another tenebrionid beetle Alphitobagus bifasciatus (Lockey, 1979). n - Nonacosene (double bond position unknown) is the second most abundant n - alkene of the louse, and monounsaturated alkenes containing 29 carbon atoms have been identified in the cuticular hydrocarbons of several other insect species including T. molitor, T. obscurus (Lockey, 1978a), A. bifasciatus (Lockey, 1979) and P. japonica (Jackson, 1972).

The absence of terminally branched monomethylalkanes in the cuticular lipids of the louse is most unusual since the greater majority of arthropods investigated so far possess either 2 - methylalkanes or 3 - methylalkanes or both (Lockey, 1980a). It may be possible, however, that terminally branched monomethylalkanes are present in the louse in extremely low concentrations and are not readily resolved during gas chromatography.

Internally branched monomethylalkanes (12.1%) do not comprise an unusually high proportion of the louse hydrocarbons in comparison with those of other insect species, and the composition of the series ( $C_{24}$  to  $C_{34}$ ) is strictly conventional with the 11 - and 13 - methylalkanes predominant (Nelson, 1978). Although dimethylalkanes are present in the cuticular hydrocarbons of the louse, their percentage of the total hydrocarbons is low (about 4.7%) compared to that in other insects where they occur.

Hadley (1978, 1981) likens the surface wax layer on arthropod cuticle to a plasma membrane or an artificial bilayer and studies involving these two systems indicate that permeability to water is increased by the presence of unsaturated or branched molecules (de Grier et al., 1968; Taylor et al., 1975) and is decreased by the addition of larger molecules (de Grier et al., 1968). It has been noted that among arthropods, a high content of unsaturated or branched chain hydrocarbons in a cuticular wax tends to lower the melting temperature of the mixture and increases its mobility (Gilby and Cox, 1963; Neville, 1975; Baker et al., 1978). The predominantly unsaturated hydrocarbon mixture from the cockroach P. americana melts at a temperature  $10^{\circ}\text{C}$  lower than the respective cuticular hydrocarbon mixtures from the cockroaches Leucophaea maderae and Blattella orientalis which contain only saturated constituents (Tartivita and Jackson, 1970). That the differences in melting point temperatures might be associated with the climatic conditions which the cockroaches inhabit, such as temperature and

humidity, is supported by observations on the naiad and adult forms of the big stonefly Pteronarcys californica (Arnold et al., 1969) and on larvae and adults of the black carpet beetle Attagenus megatoma (Baker et al., 1979a). In both species, the differences in surface lipid composition between the two developmental stages which are characterized by the presence of unsaturated hydrocarbons in the naiad P. californica and the adult A. megatoma reflect the varying needs for water conservation (Arnold et al., 1969; Baker et al., 1979a). In addition, the fact that the cuticular hydrocarbons from many desert species of arthropods such as the scorpions Hadrurus arizonensis (Toolson and Hadley, 1977) and Paruroctonus mesaensis (Hadley and Jackson, 1977), the locust Schistocerca gregaria (Lockey, 1976) and the five tenebrionid beetles Eleodes armata, Cryptoglossa verrucosa, Centroniptera muricata, C. variolosa and Pelecyporus advereus (Hadley, 1978) are completely saturated supports the concept that unsaturated cuticular hydrocarbons occur in those arthropods inhabiting environments in which transcuticular water loss is not a problem. As was established earlier, the lice used in these studies were reared under conditions where the potential for desiccation was negligible and there appears to be a correlation between this and the high overall content in the cuticular hydrocarbons of unsaturated and branched chain constituents.

The remaining lipids in the cuticular wax of the louse, cholesteryl esters, triglycerides, fatty acids, 1, 3 - diglycerides and sterols (cholesterol) are present in roughly equivalent proportions and no single class would be expected to have a significant effect on the overall properties of the wax, particularly in view of the abundance of the hydrocarbons. The general predominance of palmitic acid and oleic acid in the lipid classes except sterols is common among insect cuticular waxes (Jackson and Blomquist, 1976). A cholesteryl ester which constituted the second most abundant non-hydrocarbon component after cholesterol in the louse also contained the longest fatty acid from the lipid classes. The medium chain length (25 carbon atoms) of the cholesteryl ester fatty acid is in keeping with a soft cuticular wax in the louse rather than a hard one (Baker *et al.*, 1979a).

Quantitative analyses of the whole body and faecal lipids of the louse were carried out by thin-layer chromatography/densitometry because only small samples were available while the cuticular lipids were analysed using the same method for comparative purposes. Although there was poor numerical agreement between the cuticular lipid composition obtained by this method and that obtained by weighing the lipid classes, the hydrocarbons constituted by far the major fraction in both analyses. The abundance of fatty acids in the whole body lipids corresponds with their importance in

insect nutrition (Gilbert, 1967) and the comparatively high levels of cholesterol and cholesteryl esters in the whole body lipids and the faecal lipids are consistent with the essential requirements of insects for cholesterol and its steady supply in the diet (45 mg/100 ml rabbit's blood or 0.3  $\mu$ g in 0.75  $\mu$ l; Spector, 1956).

Although earlier studies on the grasshopper Melanoplus sanguinipes have demonstrated that n - alkanes may be obtained directly from the diet (Blomquist and Jackson, 1973), this is unlikely to occur in the louse since long chain hydrocarbons are not present in its diet (rabbit's blood) in appreciable quantities. The extensive incorporation of labelled oleic acid into the hydrocarbon fraction of the cuticular lipids suggests that oleic acid might be a natural precursor of these hydrocarbons and since the louse lipids were collected 24 hours after ingestion of the radiolabelled material, the high level of incorporation implies that the surface wax turnover is quite rapid. In the lipids of the whole body and the faeces, the low level of incorporation of radiolabelled material into cholesterol compared with that into the cholesteryl esters is consistent with most of the cholesterol being derived directly from the diet.

The situation in which the hydrocarbons in the faecal and cuticular lipids of the louse are qualitatively and quantitatively similar has also been observed in the adult housefly Musca domestica

by Silhacek *et al.* (1972). Although they proposed that the faecal matter becomes contaminated either in the gut, or, externally through the condensation of hydrocarbons which have evaporated from the cuticle surface, in the louse it probably occurs simply by contact between the faeces and the surface lipid layer.

The quantitative differences between the hydrocarbons in the cuticular wax and the (male and female) whole body lipids may have resulted from the incomplete removal of cuticular lipids from the bodies of the lice. However, the fact that *n*-nonacosane, a major cuticular hydrocarbon, was detected in only trace amounts in the whole body hydrocarbons suggests that efficient extraction of cuticular lipids did occur prior to the analysis of the whole body hydrocarbons. The occurrence of large quantitative differences between the male and female hydrocarbons from whole body extracts of lice appears to be in conflict with the original findings which showed the cuticular hydrocarbons from male and female lice to be virtually identical (see section 3.2.4.1.). Male and female lice used in the analyses of cuticular hydrocarbons were selected from colonies in which there was repeated contact between all insects. A transfer of cuticular waxes during these encounters might explain why the male and female lice possessed similar cuticular hydrocarbon distributions. The higher levels of *n*-heptacosane, *n*-octacosane and 15-

hentriacontene in the male whole body hydrocarbons is curious, but may be connected with a similar phenomenon observed in the haemolymph of the male cockroach P. americana whereby the fluctuation of hydrocarbons varies with photoperiod (Turner and Acree, 1967).

#### 4.5. Applied aspects.

##### 4.5.1. Cuticular permeability/transition temperature.

Although some doubts have been raised concerning the existence in terrestrial arthropods of a 'transition temperature' or a specific temperature at which an abrupt transcuticular water loss takes place (Toolson, 1978), the data from my studies suggest that this phenomenon does occur in the louse. The upper lethal temperature of the louse ( $50-52^{\circ}\text{C}$ ) and the melting temperatures of its cuticular wax ( $41-42^{\circ}\text{C}$  and  $49.5-50.5^{\circ}\text{C}$ ) are consistent with observations on the change in electrical potential across the cuticular surface of female lice with increasing temperature. Recorder traces of this change indicate that electrically conductive material, presumably water, is deposited slowly on the cuticular surface above  $46-47^{\circ}\text{C}$  and very rapidly above  $48-49^{\circ}\text{C}$ . This also agrees with my observation that lice of both sexes become covered with a film of moisture immediately prior to the thermal death-point. Wigglesworth (1942) found that when lice were held under olive oil for long periods, droplets of water were exuded from the surface of the cuticle and appeared primarily in areas of sclerotisation. This corresponds with the results of my ultrastructural studies which showed that sclerotised louse cuticle contains significantly more epicuticular filaments reaching the surface than does membranous louse cuticle.



The upper lethal temperature for lice of  $46.5^{\circ}\text{C}$  reported by Mellanby (1932) was obtained from trials in which the various temperatures were maintained for 1 hour. Under these circumstances, death probably resulted from slow desiccation as opposed to the rapid water loss at the higher lethal temperature range ( $50 - 52^{\circ}\text{C}$ ) obtained in my experiments. That the cuticular surface of the male adult louse is electrically conductive at ambient temperatures (see section 3.3.1.3.) is unusual but might be explained by the fact that the rate of cuticular transpiration in these is probably high as a result of being reared under conditions where desiccation is not a problem (see section 4.2.). This explanation is, however, contradicted by the fact that the cuticular surface of the female adult louse is electrically non-conductive at ambient temperature.

On the basis of his observations on permeability change with temperature Beament (1945) proposed that a highly oriented monolayer of polar lipids at the interface of the epicuticle and surface lipids controls the passage of water through the cuticle. He concluded that the sudden increase in permeability associated with the transition temperature results from the disorganisation of the oriented monolayer. In an alternative theory, Locke (1965) suggested that the epicuticular filaments observed in insect cuticle are lipid-water liquid crystals and phase changes within these structures may be responsible for the variable permeability of cuticle to water. Filshie (1970 a, b), however, found that the

epicuticular filaments of Calpodex ethlius and Lucilia cuprina are formed substantially of stabilised lipids since they are unaffected by acid hydrolysis and lipid solvents.

#### 4.3.2. Toxicity test procedure.

The most commonly employed technique for evaluating the toxicities of various compounds to lice involves the exposure of insects to squares of cloth or filter paper impregnated with the toxicant in the form of a dust or an acetone solution (Busvine, 1971). In tests of this type, the insects are usually kept in darkness at a constant temperature ( $25^{\circ}\text{C}$ ) for a period of 24 hours before determining the rate of mortality. It is obvious that there are many variable factors associated with this procedure which is regarded only as a semi-practical test (Busvine, 1971). In an effort to eliminate as many variables as possible, it was decided to use the topical application technique since it involves giving known doses to individual insects. Attempts to restrain lice for testing by placing them upright on double-sided Sellotape were unsuccessful due to the effects of some of the test compounds upon the Sellotape. With the migration of the organic liquids over the surfaces of the lice, glue material in contact with the lice was soon reached and dissolved which allowed the insects to escape. Placing individual lice in separate containers (see section 2.3.3.2.) was more successful

but as the results indicate (see section 3.3.2.1.), the choice of container is critical. Of the three types of containers tested, the glass container gave the most satisfactory results on the basis of reproducibility.

The internal surfaces of the three types of containers were examined under UV radiation after having held lice dosed with Rhodamine B dissolved in 2-dodecanone for 24 hours. In the case of the plastic containers, much of the test solution applied to the lice became deposited on the internal surfaces of the containers whereas the surface of the glass containers was essentially unwettable.

Taking an objective view of the toxicity test procedure used in my studies, the principal advantage lies in the reproducibility of results but must be weighed against the fact that the procedure, in its entirety, is particularly time-consuming.

#### 4.3.3. Toxicity of potential insecticide solvents.

In many arthropods, treatment with various toxicants causes a marked increase in the water loss rate by upsetting processes involved with water conservation (Ebeling, 1976). Both Wigglesworth (1942) and Beament (1945) have provided evidence that oils and organic solvents cause significant water loss from insects by

disruption of the epicuticular wax layer and this is of particular relevance in the present studies. Of the ten potential insecticide solvents investigated, 1,4-butanediol and 1, 2-propanediol were least toxic to lice and were also the only solvents in which the simulated cuticular wax mixture did not dissolve. These results are consistent with those from the cuticular penetration studies using solutions of Rhodamine B (see section 3.3.4.). Four hours after topical application of a 0.5% solution of Rhodamine B in 1, 4-butanediol, weak fluorescence was observed on the surface of the louse cuticle only (Plate 56), whereas a solution of Rhodamine B in the more lipophilic solvent, dodecanol, produced fluorescence throughout the procuticle (Plate 55), indicating extensive penetration by the solution.

The remaining eight solvents appear to form two separate groups based on their levels of toxicity to female adult lice (Figure 47). In general, solvents of the less toxic group were more efficient in dissolving the simulated cuticular wax mixture than those of the more toxic group, and the toxicity of the former probably results solely from the disruption of epicuticular wax. The higher toxicity of the more toxic group of solvents arises from other properties such as the possession of more favourable phase partition coefficients (Webb and Green, 1945; Burt and Lord, 1968; Burt et al, 1971) in addition to wax solubility. Significantly, the most toxic of the ten solvents, lauryl ether, is the only one readily miscible with water and this property allows it to diffuse

from the lipid phase of the outer cuticle to the aqueous phase of the interior of the insect. The penetrative and toxic nature of lauryl ether appears not to be significantly impaired when it is applied as an aqueous solution. Being the less volatile of the two liquids, lauryl ether remains on the cuticular surface as the water evaporates.

In view of the preceding results, indicating the ability to dissolve the epicuticular wax layer and partition from lipid phase into aqueous phase, lauryl ether appears to have the most potential as an insecticide carrier. Limited tests with dieldrin in lauryl ether suggest that this is correct but a more thorough investigation is necessary before firm conclusions may be drawn.

#### 4.3.4. Tracer studies with solutions of Rhodamine B.

Whilst the results of the penetration studies using Rhodamine B in either lauryl ether or dodecanol are consistent with the theory that insecticides and solvents pass through the cuticle via the pore canals and into the haemolymph (Plate 55) (Wigglesworth, 1942; Lewis, 1965), there is also some evidence of entry via the tracheal system (Plate 53) (Gerolt, 1969, 1970). As far as the cuticular route is concerned, heavier Rhodamine B staining in sclerite cuticle than in membranous cuticle does not necessarily indicate that the main path of entry occurs in the former type of cuticle. Nevertheless,

earlier experiments by Wigglesworth (1942) suggest that the sclerite cuticle of the louse is more permeable than the membranous cuticle and this is consistent with the results of my ultrastructural studies which indicate that surface pores are more numerous on sclerite louse cuticle than membranous. Furthermore, in some micrographs of Rhodamine B - stained sclerite cuticle, a striated effect is evident between the epidermis and the epicuticle (Plates 51 and 55) which may be attributed to the presence of pore canal structures. Lewis (1963) however, has demonstrated that penetration is possible through cuticle in which pore canals and dermal gland ducts are absent and some degree of lateral diffusion through the louse cuticle might therefore be expected. The role of Rhodamine B as a protein stain (Nairn, 1976) and its differential staining of sclerite and membranous cuticles is discussed more fully in section 4.1.4.

Gerolt (1969, 1970) suggests that contact insecticides enter an insect by a lateral migration through the cuticle (endocuticle) into the tracheal system. However, the Rhodamine B fluorescence in the trachea in Plate 51 has probably resulted from surface migration of the solution (Lewis, 1962) rather than lateral diffusion in the cuticle as proposed by Gerolt.

The shortcomings of this method of tracing penetration in insect cuticle are fully recognised; but while there is almost certainly a difference in the rates of penetration between Rhodamine B and the

test solvents, it is improbable that the stain could diffuse through the cuticle unless aided by a solvent. Autoradiography, which is a more informative method, could not be used in this case however, since the time available was limited.

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## Appendix.

Krebs - Ringer solution (  $\text{Ca}^{++}$  omitted ).

## ( Intermediate solutions )

1.	0.90% NaCl	(0.154M)
2.	1.15% KCl	(0.154M)
3.	2.11% $\text{KH}_2\text{PO}_4$	(0.154M)
4.	3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	(0.154M)

## Intermediate solutions mixed as follows:-

1.	100 parts
2.	4 parts
3.	1 part
4.	1 part